THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF ENTOMOLOGY

QUALITATIVE ANALYSIS OF EFFECTS OF FORMULATION ADDITIVES ON METABOLISM OF CHLOROTHALONIL IN HONEY BEES

DAVID G. BAHN
Spring 2011

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Secondary Education
with honors in Entomology

Reviewed and approved* by the following:

Christopher A. Mullin
Professor of Entomology
Thesis Supervisor

Diana Cox-Foster
Professor of Entomology
Honors Adviser

* Signatures are on file in the Schreyer Honors College.

Abstract

Two groups of honeybees were exposed to orally administered, low level doses of formulation and technical grade chlorothalonil (approximately 2µg/bee/day) continuously over a three day period. Excreta samples were collected every 24 hours, along with mortality data. Following collection, excreta were extracted, and samples analyzed using LCMS-UV/Vis spectroscopy. In two separate trials, the mortality rate among the group that received the formulation dose was at least four times higher than the technical and control dose groups. A significantly higher mortality rate was observed during the 24-48 hour time period than during the 48-72 hour time period. Photodiode array (PDA) data demonstrates significant differences in the chemical profile of excreta samples collected at 24 and 48+ hours, suggesting a correlation with observed mortality. PDA and LC-MS data also confirm the presence of chlorothalonil in collected wax chips.

Table of Contents

Acknowledgements

The author would like to thank Dr. Christopher A. Mullin for his full cooperation, assistance, and guidance in all aspects of this project, particularly experimental setup, data analysis, and proofreading of the final paper. Additional thanks to Dr. Diana Cox-Foster for helpful suggestions and final proofreading. Also, thanks to Ms. Sara Ashcraft for her help in the lab, especially experimental setup and collection of bees for all experimental trials.

The author would also like to express gratitude to Maryann T. Frazier for the initial introduction that made the project possible, and to Dr. James L. Frazier for his contributions to early discussions of topic selection.

The author would also like to thank Drs. Frazier and Mullin for providing access to Department of Entomology lab facilities (520 Agricultural Sciences Building) and instruments (most notably a Shimadzu LCMS 2020) for the duration of the project.

Introduction

Current investigations into Colony Collapse Disorder (CCD) are focusing on the role various agricultural chemicals may play in contributing to colony stress. Due to the lack of dead bees that characterizes CCD, as well as the large number of pesticides and fungicides discovered in beeswax and pollen samples, many recent lines of inquiry are focused around the sub lethal effects of chemical exposure, as well as synergistic effects of various chemical agents at low levels.[1] Such studies involve exposing insects to low levels of chemical agents, approximately the dose that causes mortality of 1-5% of the test population (LD 1-5), and often focus on behavioral effects. Working with such low exposure levels poses a number of challenges, principally understanding what constitutes a low level dose. Current doses are estimated using LD 50s determined for various chemical agents on honey bees.

LD 50s are generally determined from probit analysis of lethal responses over a range of doses of an active ingredient, giving rise to rapid mortality (2 days or less). This method, then, most closely models a single, acute exposure in the natural environment. One common example would be spraying a field of cantaloupes to kill cucumber beetles. The spray is applied directly to the insects at a high dose of chemical agent, resulting in illness or death. While determining LD 50s by this method may be accurate for modeling such exposure routes, it is not an accurate representation for all chemical exposures. Honey bee hives, specifically, are generally kept somewhat apart from the crops they are used to pollinate, and not often subjected to direct chemical spraying (unless the keeper is treating for mites or other parasites). While some bees may die as a result of acute exposure to chemical agents during application, the majority are not dosed directly, but feed on contaminated pollen and nectar, consequently receiving a smaller dose orally, rather than an acute dose by contact.

This low level oral exposure continues until rain or degradation over time reduces the concentration of chemical agents in the foraging area to a negligible level, assuming the chemical agent is not reapplied. In a 2001 paper, Potter et al. cite a 1-3.5 day half-life for chlorothalonil in the soil of peanut fields following application.[2] Given this evidence, it is likely that such low level doses occur for several days following a chemical application. Since many pesticides require regular reapplication, it is reasonable to assume that foraging activity regularly results in low level exposures of chemical agents.

Given the apparent realities of low level, oral, multiple day exposure, dose levels based on an acute LD 50 are not likely to be representative of a compound's true sub-lethal effect on the population of a hive. Given the widely accepted role of chemical agents in contributing to CCD, a more accurate model for predicting dose levels for sub-lethal effects from such indirect, low level chemical exposure is crucial to understanding, and hopefully combatting, Colony Collapse Disorder.

Another major concern regarding dose determination for low level exposure is the issue of commercial formulations. Current legislation requires a company to register and test only new active components with limited testing of the chemical formulations actually applied in the field. Subsequent chemical formulations containing those active ingredients in combination with surfactants and other additives, are not subject to mandatory testing for safety to non-targeted organisms. Recent research conducted at Penn State and elsewhere has shown synergistic effects in formulations of combined active ingredients on beneficial insects, such as honey bees. In addition, the use of surfactants to increase solubility of non-polar active ingredients in water (as a solvent for commercial application) may well result in more systemic effects than the application of a technical compound with a single surfactant. Given these realities, it is possible that

exposure to commercial formulations may result in a different metabolite profile than exposure to any single active ingredient. This could result in observable differences in effects from acute exposure, as well as sub-lethal effects from long term, low level exposure, and further complicates determination of dose levels where sub-lethal effects are observable.

With these challenges in mind, the following work attempts to provide evidence to answer the following major questions:

1) Will a technical grade active ingredient display the same lethality as a formulation containing that active ingredient at equivalent concentrations in a prolonged low level oral dose?

2) Will the metabolite profile from oral dosing of an active ingredient vary over time in a prolonged dose?

3) Does oral dosing of a technical compound result in the same metabolite profile as oral dosing of a commercial formulation at similar dose levels?

While beneficial insects face exposure to large numbers of chemical agents, for the following work it was necessary to select a model agent. 2,4,5,6-tetrachloroisophthalonitrile (chlorothalonil) was chosen for a number of reasons. Chlorothalonil is a broad spectrum fungicide active against a wide range of phytopathogenic fungi. In a 1968 study involving *Saccharomyces pastorianus* and *Neurospora crassa*, Vincent and Sisler provide evidence suggesting thiol deactivation as the primary mechanism of fungicidal action for chlorothalonil.[3]

In a 2010 study, Mullin et al. conducted analysis of samples from migratory and other beekeepers, in total comprising colonies from 23 states and one Canadian province.[1] Chlorothalonil was found in 49.2% of wax samples (up to 53ppm) and 52.9% of pollen samples

(up to 99ppm).[1] These data demonstrate that chlorothalonil is a widely used fungicide that is present at high concentrations in many bee hives. In a similar study in 2009, vanEngelsdorp et al. report finding chlorothalonil in 100% of "entombed pollen" samples, a phenomenon associated with colony mortality.[4]

Chlorothalonil was also selected due to the reasonably well known chemistry of its environmental and biological breakdown. The chemistry of chlorothalonil centers primarily on halogen displacement in reactions with alkyl thiolates, such as glutathione, though chlorothalonil is also vulnerable to attack by a variety of nucleophiles (including hydroxide in basic conditions), especially at carbon atoms ortho-positioned to activating cyano groups.[5] Figure 1 illustrates some of these enzymatic and chemical changes.

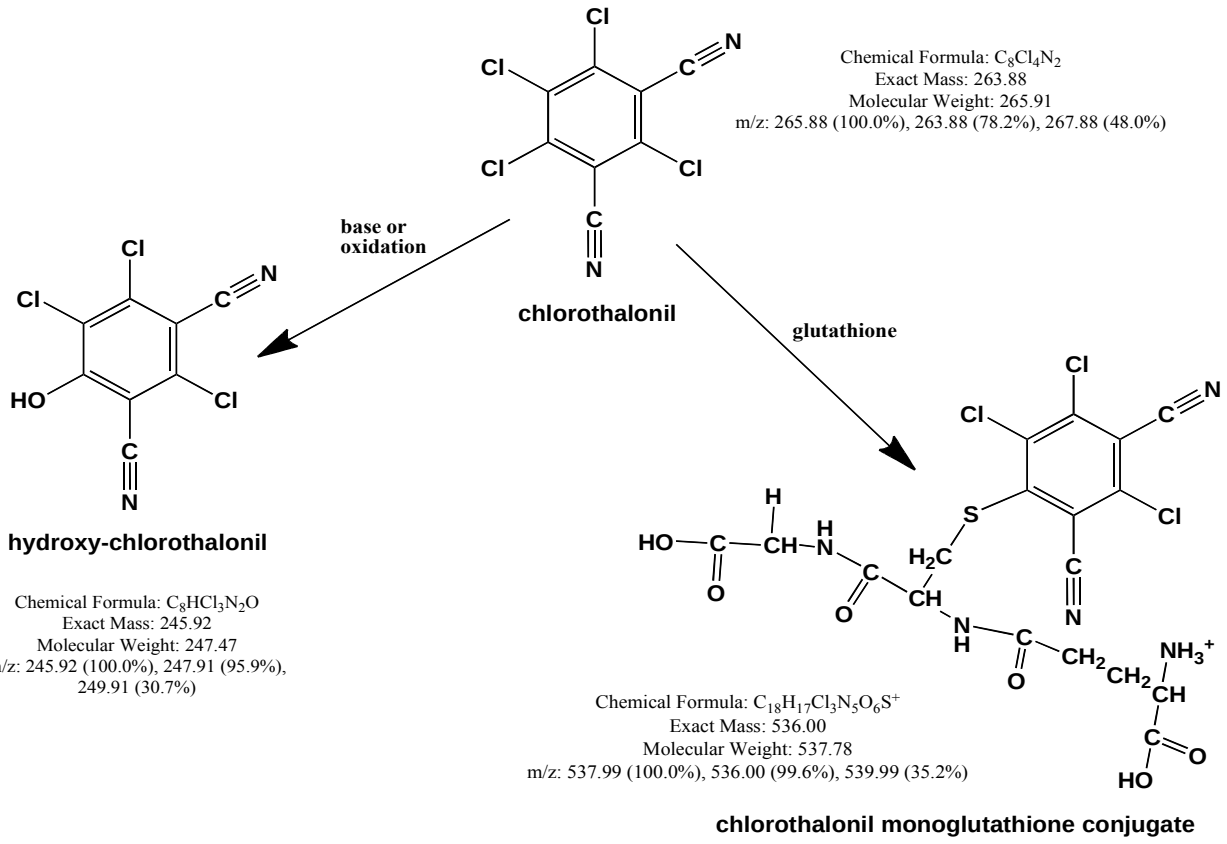


Figure 1: The major enzymatic and chemical reactions of chlorthalonil with base, glutathione, or enzymatic oxidases.

While it is recognized that a large number of beneficial organisms are routinely exposed to a wide variety of chemical agents, for the course of this work it was necessary to select a model insect. Honey bees were chosen in the hope that collected data and results of this work, utilizing a chemical agent associated with colony mortality (vanEngelsdorp, 2009), may contribute to future research undertaken to understand and combat Colony Collapse Disorder.[4]

Analytical Methods

Lethality data was collected by direct observation of cages of bees over a three day period and counting the number of dead and moribund bees every 24 hours (see Biological Methods on page 11). Analysis for chlorothalonil metabolites presented a greater challenge.

Several pathways for chlorothalonil metabolism are known in various plants, animals, and fungi which could provide a basis for investigating the pathways of this fungicide in honeybees. While detailed knowledge about metabolism pathways will no doubt prove crucial in understanding the effects of formulation additives on the metabolism of chemical agents, such specific investigations are beyond the scope of this work. Rather, the goal was to introduce a chemical agent in a technical and commercial formulation, and look for possible differences in the metabolite profile. The goal of detoxification pathways is to convert toxic substances into molecules that can exit the body via waste disposal systems. If formulation additives cause differences in final metabolites, the excreta of bees dosed with technical active ingredient and a formulation containing that active ingredient should have a different chemical composition. Using a small trial group, a system for collection of excreta was devised by covering the bottom of an incubation box with filter paper. Excreta could then be characterized by daily observation, and collected for chemical analysis.

Chemical analysis of samples was conducted using a Shimadzu® LCMS 2020 equipped with UV/Vis photodiode array (PDA) and electrospray ionization (ESI) detectors. A Mass Spectrometer (MS) is a widely used instrument for the analysis of chemical samples for identification of components and detection of impurities. A sample mixture is injected and resolved on a high-pressure liquid chromatographic (HPLC) column using a solvent gradient. The solvent stream then enters the PDA detector followed by the ESI-MS detector where it is

vaporized. Molecules in the gas phase are then bombarded with a high voltage electron beam, which causes fragmentation into ions with a mass to charge ratio (m/z). The gas phase ions are then passed through a magnetic field of known strength, causing deflection, which allows the separation of the fragments according to their mass to charge ratio. This signal is then detected and amplified, and can then be interpreted as a table of m/z values given in relative abundance. Since identical samples display consistent m/z fragment composition, mass spectroscopy readouts of m/z values for a sample are an invaluable tool for identification of chemical compounds.[6]

If a sample contains several different chemical species, separation is necessary before useful mass spectroscopy data can be acquired. Generally, some form of chromatography is used to separate the components of the sample based upon polarity. Gas phase chromatography is used in Gas Chromatography Mass Spectroscopy (GCMS), when samples can be injected as gases, and an inert carrier gas such as helium or nitrogen is used as the mobile phase. However, many metabolites and parent compounds of interest do not have sufficient vapor pressures to make injection as a gas sample feasible. Recently, analysis of such samples has been conducted using Liquid Chromatography Mass Spectroscopy (LCMS). This technique involves injection of a liquid sample and the use of a liquid mobile phase to separate compounds in the sample based on polarity. Once separation is achieved, the sample is vaporized and analyzed via a standard mass spectroscopy procedure.[6]

Other useful data was collected in the form of UV/Vis spectroscopy. This technique makes use of distinctive energy transitions that occur between excitation states of electrons in molecules. A light source (generally ranging from 200-800nm) is used as the radiation source in the instrument. A monochromator is used to filter out all light except a certain wavelength. This

wavelength of light is passed through a sample in liquid phase and absorbance readings are recorded. The process is then repeated with a different wavelength of light slightly different from the first, and a new absorbance value is obtained. This process is repeated over the range of wavelengths specified, and detection with a PDA allows the creation of an absorption profile for a compound over the entire range of wavelengths, with an often distinctive wavelength of maximum absorption (λ_{max}). Since different classes of compounds will have different absorption spectra, this method is widely used to identify chemical compounds as a cheaper alternative to a more expensive analysis like mass spectroscopy.[7]

When used in conjunction with a chromatography method, it is possible to use UV/Vis spectroscopy to detect a large number of chemical species within the same sample based upon their different absorption profiles, even if definitive identification based on this data alone is difficult. If a number of scans are taken at varying known concentrations of a certain species, it is even possible to use UV/Vis spectroscopy to determine the concentration of a particular species in a sample by using integration to determine the area under the curve created by the absorption peak.[7] Such quantitative methods, though regularly utilized, are not the focus of this study. Rather, UV/Vis spectroscopy will be utilized as the primary method for investigating possible differences between excreta of technical dosed bees and formulation dosed bees based upon the absorption profile of the sample following separation using HPLC.

Once analytical methods were chosen, it was necessary to begin refining instrument parameters. Chlorothalonil is a relatively non-polar molecule, with many of its metabolites and degradation products being significantly more polar than the parent compound.[8] A model system was necessary to test the settings on the LCMS to insure satisfactory separation of possible

metabolites present. As chlorothalonil metabolism by glutathione conjugation has been demonstrated in tomatoes by Wang et al.[9] , in soil microbes by Kim et al.[10] , in several fish species by Davies[11] and Gallagher et al.[12], and in rats by Hillenweck et al.[13], the use of glutathione conjugates as a model sample mixture for the possible metabolites of chlorothalonil in honey bees is likely a suitable model system.

Using methods described by Hamersak, glutathione conjugates were synthesized.[14] 7.59 milligrams (mg) of analytical standard grade chlorothalonil were dissolved in 7 milliliters (ml) of solvent grade acetonitrile. A magnetic stir bar was added to this solution, and the solution was stirred on a low setting. Three stoichiometric equivalents of glutathione were dissolved in 2 ml of distilled water and added drop wise to the chlorothalonil solution. A precipitate was observed that dissolved upon further stirring. At this point, pH paper showed the pH of the chlorothalonil-glutathione mixture to be between 3.5 and 4. An alkaline solution was made by dissolving three stoichiometric equivalents of sodium carbonate in 1ml of distilled water. The entire stoichiometric amount of alkaline solution was added drop wise to the reaction mixture to promote deprotonization of the glutathione acid group.[14] Following addition of alkaline solution, pH paper indicated the pH of the reaction mixture to be between 6.5 and 7. Reaction mixture was covered with aluminum foil and stirred for 30 minutes. At this point samples were withdrawn and subjected to LCMS analysis.

Using this sample mixture, a two solvent system gradient using acetonitrile and water was developed that would provide acceptable separation of conjugates, starting with a high percentage water polar phase and gradual shift to higher acetonitrile content over 12 minutes to allow separation of more non-polar metabolites and the parent compound.[8] After several iterations, it was decided to utilize a gradient that began at 5% acetonitrile, 95% water.[8] Isocratic

conditions were maintained for two minutes after injection of a 1-4µl sample. At this point, the percent of acetonitrile was increase until 12 minutes, at which point the gradient consisted of 95% acetonitrile, 5% water. Isocratic conditions were maintained for two minutes, at which point the percentage of acetonitrile was decreased over two minutes to initial percentages. Isocratic conditions were then maintained for the remaining four minutes of the run (22 minutes total). This solvent system was used for all data analysis conducted on experimental samples in an attempt to allow some comparison between samples based on retention times.

Resolution of the mixture was achieved on a Shimadzu Shim-Pak XR-ODS 2.0 X 100 mm column thermostatted at 40° C. By spiking a sample with a 100ppm analytical standard, chlorothalonil was assigned a retention time of 12.10 minutes in the UV/Vis spectrum and roughly 12.20 in the mass spectroscopy profile. A reference spectrum was also obtained, showing a λ_{max} value of 232nanometers (nm) with a shoulder at 255 nm. These values are in agreement with spectral values published by Hamersak et al. for chlorothalonil.[14]

Biological Methods

With the challenges discussed in the introduction in mind, great consideration was given during experimental setup to model the conditions most likely for indirect exposure to honeybees. All bees were dosed orally at similar concentrations to those reported by Mullin et. al. in contaminated pollen samples, namely 100 parts per million (ppm) chlorothalonil.[1] As chlorothalonil is not readily water soluble at these concentrations, a surfactant was necessary to achieve the desired concentration.[15] This necessitated the addition of a control group that would be dosed with the chosen surfactant alone. Due to availability and use in prior work, 1-methyl-2-pyrrolidinone (1M2P) was selected.

Solutions were prepared fresh daily to prevent side reactions. Technical solutions were stored in sealed vials in a refrigerated environment. Doses were delivered via 6 x 30 mm glass tubes that contained roughly 400 µl with an open end covered by a 120 micron nylon mesh. To allow for mishaps, 2ml of each solution was prepared per day of the trial. Solutions were prepared as follows. All micro liter measurements were accomplished with micro pipettes and replaceable plastic pipette tips.

Control Treatment (1% 1M2P): Mix 1980 µl of 1:1 sucrose: water solution with 20 µl 1M2P in a new glass test tube. Vortex for 1 minute. Draw off 400 µl of solution into clean pipette tip. Fill glass tubes for treatment (400 µl each). Cover tube with mesh, seal edges of mesh with parafilm wrap. Weigh each tube before placing in cages.

Technical Treatment (100ppm solution in 1% 1M2P): Dilute 20 µl of 10,000ppm chlorothalonil (solvent 1M2P) in 1980 µl of sucrose: water solution in a new glass test tube. Vortex for 1 minute. Draw off 400 µl of solution into clean pipette tip. Fill glass tubes for treatment (400 µl

each). Cover tube with mesh, seal edges of mesh with parafilm wrap. Weigh each tube before placing in cages.

Formulation Treatment (100ppm Bravo Weatherstick®): Dilute 35 µl Bravo Weatherstick® (56% chlorothalonil as purchased) in 2000 µl 1:1 sucrose: water solution in a new glass test tube. Vortex for 1 minute. Draw off 400 µl of solution into clean pipette tip. Fill glass tubes for treatment (400 µl each). Cover tube with mesh, seal edges of mesh with parafilm wrap. Weigh each tube before placing in cages.

All bees used in this work were house bees taken from Department of Entomology hives in early June 2010 (June 1, and June 7, for first and second trial, respectively). Bees were anaesthetized using refrigeration and divided into cages with 20 bees per cage. Each cage was labeled with the letter of a treatment group. Each treatment group comprised 4 cages of 20 bees each for a total of 80 bees per treatment group. Group A was selected as the control group, which received a 1% solution of 1M2P in a 1:1 sucrose: water solution daily. Group B was selected as the technical dose group, which received a 100ppm solution of technical chlorothalonil in a 1:1 sucrose: water solution with 1% 1M2P as a surfactant daily. Group C was selected as the formulation dose group and received a 100ppm solution of Bravo Weatherstick® in 1:1 sucrose: water solution daily.

Each treatment group was placed into a plastic incubation box lined with filter paper. Initial treatments were administered, and the boxes were placed in a growth incubator kept at 30^{o}C in the dark. Treatment groups were allowed to incubate for 24 hours, then removed from the incubator for observation. Glass feeding tubes were swapped for new tubes containing the

appropriate treatment, and masses of old tubes were recorded. Mortality data was also recorded, along with observations about amount and type of excreta present. Excreta samples were monitored by counting the number of spots to appear on the filter paper in a 24 hour period and characterized as either chunks or smears based on appearance.

Excreta samples were collected by two main methods. If a large volume of dried material was present, the sample was scraped with a pair of tweezers into a weighing boat, and transferred into a glass vial for storage. For small amounts of excreta that had absorbed into the filter paper, the sample was cut from the filter paper leaving a roughly 1 mm ring of white paper around the sample. Samples were stored in sealed vials and frozen at -20^{o}C for future analysis.

Two trials were conducted using the previously described procedure, begun 1 week apart. Trial 1 was initiated on June 1st 2010. After receiving three treatments (72 hours of incubation) the trial was terminated by freezing, and excreta was removed and collected for analysis. Collected wax chips were observed in the first trial, and were collected using tweezers cleaned in ethanol. Collected wax chips were also frozen for future analysis. Trial 2 was initiated June 7th 2010. Excreta were removed every 24 hours and frozen for future analysis. After 72 hours of incubation, the trial was terminated by freezing and final samples were removed for analysis.

All samples were extracted using a 65% acetonitrile/34% water/1% glacial acetic acid extraction solvent. To facilitate qualitative comparison between collected PDA data in future analysis, it was important to use approximately equal masses of excreta from different treatment groups. To achieve this, 25 pieces of excreta from a single treatment were massed on an analytical balance accurate to four decimal places to determine an average mass. It was assumed that spots on filter paper would count for approximately this average mass. With these

considerations, roughly 2mg of excreta per treatment group were placed in a clean test tube and 1ml of extraction solvent was added. The sample was vortexed for 2 minutes, allowed to stand for five minutes, then pipette filtered into a clean test tube. The resulting solution was centrifuged at 2500 rpm for ten minutes. The supernatant was then removed, without further concentration or purification, into a clean glass sample vial. The vials were labeled and immediately subjected to HPLC-PDA-ESI analysis as described in the Analytical Methods section (page 6).

Results/Discussion

Mortality Data:

Mortality data collected from both trials reveal similar trends, namely greater mortality in the formulation treatment group, similar mortality in the control and technical treatment groups, and a decrease in observed mortality from 48 to 72 hours after initial exposure. Table 1 and Table 2 show total consumption of each treatment group over the course of the trial. None of the treatment groups show any appreciable deterrent effects, as all cages routinely consumed the entire offered treatment, with the exception of the final treatment given at 48 hours after initial exposure. Even in these cases, however, the amount of unconsumed treatment was not substantial, and all treatment groups consumed in the range of 1.07-1.41 grams of prepared treatment in the aqueous sucrose solution.

Table 1: Total Consumption in grams for three administered doses, Trial 1.

	Box A	Box B	Box C
Cage 1	0.31739	0.55958	0.95931
Cage 2	0.20049	0.57377	1.14264
Cage 3	0.71831	1.07657	0.97418
Cage 4	1.38001	1.30317	1.30322

Table 2: Total consumption in grams for three administered doses, Trial 2

	Box A	Box B	Box C
Cage 1	1.34760	1.31204	1.37926
Cage 2	1.19077	1.34899	1.40760
Cage 3	1.30691	1.37845	1.39893
Cage 4	1.20269	1.41493	1.41059

From this data, and the known concentrations of the treatments given, the dose per bee was calculated. This calculation was achieved by finding the amount of active ingredient consumed in each treatment per day and dividing by the total number of bees alive when the

treatment was given. These numbers were calculated daily and totaled to derive the average consumed dose of active ingredient per surviving bee at the end of the trial. Table 3 and Table 4 summarize this data for Trial 1 and Trial 2 respectively. Total received dose was on average higher for the formulation treatment groups in both trials, most likely due to higher observed mortality in the formulation treatment groups. This is consistent with observed data, since the entire offered treatment continued to be consumed despite smaller surviving numbers of bees in each cage. Received doses per bee in the technical treatment group averaged 5.8µg/bee in Trial 1 and 6.6µg/bee in Trial 2. Received doses in the formulation treatment group averaged 22.2µg/bee in Trial 1 and 8.3µg/bee in Trial 2. The given LD 50 for Bravo Weather Stick (181µg/bee) indicates that these doses should be reflective of an LD 1-5, or a dose that should cause rapid mortality of 1-5% of the treatment population.[15]

Table 3: Calculated chlorothalonil dosage per bee in µg/bee over the course of three administered doses (Trial 1), based on actual consumption of surviving bees. Shown for valid cages only (See asterisks on Table 5, page 17).

	Box A	Box B	Box C
Cage 1			
Cage 2			31.7 µg/bee
Cage 3		5.6 µg/bee	
Cage 4	0.0	6.0 µg/bee	12.7 µg/bee

Table 4: Calculated chlorothalonil dosage per bee in µg/bee over the course of three administered doses (Trial 2), based on actual consumption of surviving bees.

	Box A	Box B	Box C
Cage 1	0.0	6.3	7.1
Cage 2	0.0	6.8	7.0
Cage 3	0.0	6.4	7.4
Cage 4	0.0	6.8	11.6
Average Dose:	0.0	6.6	8.3

Actual observed mortality data is summarized in Table 5 and Table 6 for Trial 1 and Trial 2 respectively. Total observed mortality for the control treatment was zero in Trial 1 and 4 in

Trial 2. Total observed mortality for the technical treatment group was 3 in Trial 1 and 4 in Trial 2. Total observed mortality for the formulation treatment group was 32 in Trial 1, and 18 in Trial 2. After adjusting for discarded cages in Trial 1, total control population was 5 cages, total technical population was 6 cages, and total formulation treatment was 6 cages. At 20 bees per cage, these values correspond to an average of 4% mortality for the control treatment (average of both trials), 6% for the technical treatment group (average of both trials), and 42% for the formulation treatment group (average fatalities observed in both trials over three days).

While the control and technical treatment groups are well within the approximate LD 1-5 range given in the MSDS for Bravo Weatherstik®, the formulation treatment group, at 42% observed mortality, is significantly outside the anticipated mortality range. This value is even more significant considering the high rates of mortality between 24 and 48 hours after initial exposure in both trials. Since the majority of observed mortality occurred before the full treatment could be consumed, a large percentage of the reported mortality occurred at lower doses than the final reported doses in Tables 3 and 4. Since concentrations of the active ingredient and other dosing parameters were held constant, effects of other components in the formulation are likely responsible for this observed increase.

Table 5: Number dead per cage, Trial 1, Day (1, 2, and 3) from initial exposure respectively*.

	Box A (1,2,3)	Box B (1,2,3)	Box C (1,2,3)
Cage 1	12, 20, 20	13, 14, 15	6, 16, 16
Cage 2	11, 20, 20	9, 13, 14	**0, 18, 18**
Cage 3	9, 10, 11	**1, 2, 3**	11, 13, 13
Cage 4	**0, 0, 0**	**0, 0, 0**	**1, 9, 14**
Total	51	32	61
Average Per Cage	13	8	15
Total - Error Cages	0	3	32
Corrected Average Per Cage	0	1	8

*During initial dosing, an error was made and a number of feeding tubes were not accessible to the bees. This resulted in mortality due to dehydration, and this data was discarded. Bolded boxes represent valid data.

Table 6: Number dead per cage, Trial 2, Day (1, 2, and 3) from initial exposure respectively.

	Box A (1,2,3)	Box B (1,2,3)	Box C (1,2,3)
Cage 1	0, 0, 0	0, 1, 1	0, 3, 3
Cage 2	1, 3, 3	1, 2, 2	1, 1, 2
Cage 3	0, 0, 0	0, 0, 0	1, 3, 3
Cage 4	1, 1, 1	0, 1, 1	5, 10, 10
Total	4	4	18
Average Per Cage	1	1	4

One possible cause of higher observed mortality, given observed excreta, is dehydration. The formulation treatment cage consistently produced excreta that was characterized as runny (similar to regurgitate) over the course the administered treatments for both trials. In contrast, the control and technical cages produced harder, more pelletized excreta, and volumes decreased over the course of the treatment in both trials. Given these findings, it is possible that formulation components may contribute to colony stress by increasing water requirements and contributing to dehydration. Future work could investigate if the observed mortality of the formulation treatment group would decrease if water was given as part of the treatment protocol.

Another interesting trend in the mortality data was a rapid increase in observed mortality between 24 and 48 hours after initial exposure, as compared to 48 and 72 hours after initial exposure. Figure 2 and Figure 3 illustrate this trend for Trial 1 and Trial 2 respectively.

Figure 2: Mortality data for valid cages, all treatment groups, Trial 1 at 1, 2, and 3 days after initial exposure.

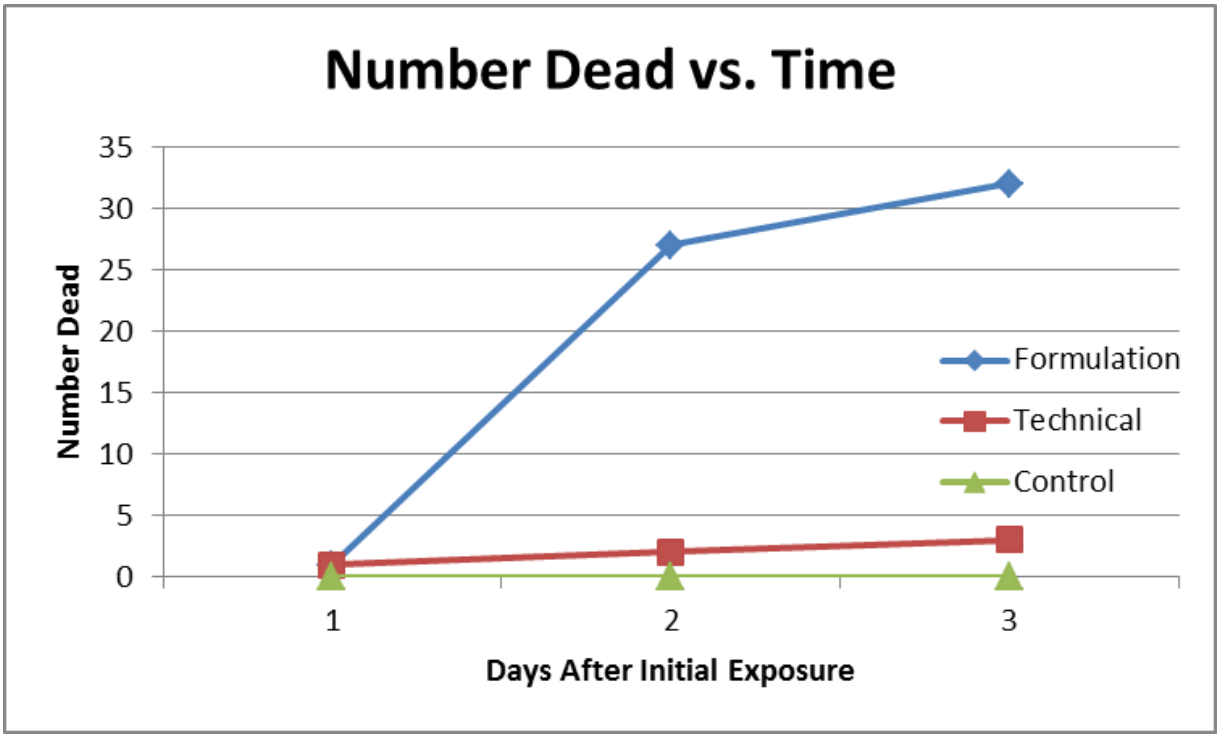
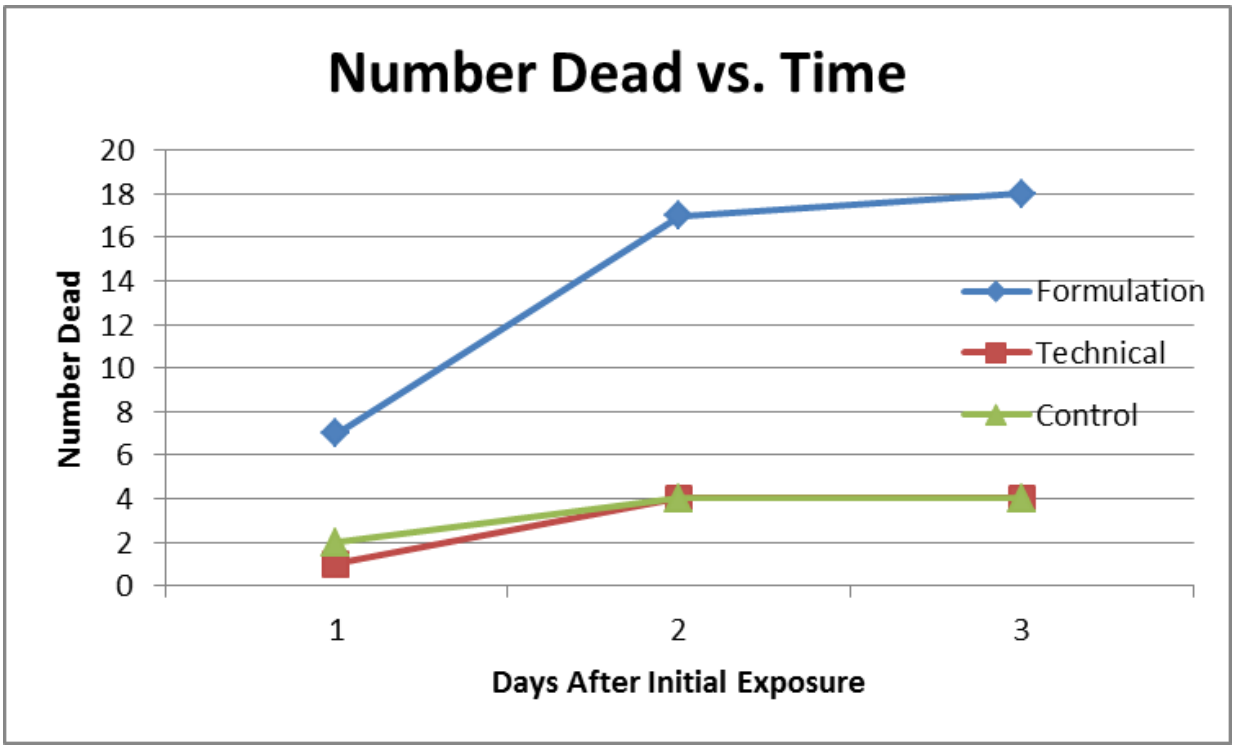


Figure 3: Mortality data for all treatment groups, Trial 2 at 1, 2, and 3 days after initial exposure.



In both plots of mortality vs. time, the graph slopes steeply from 1-2 days after initial exposure, and then levels out significantly from 2-3 days following initial exposure, rather than continuing a steady increase. This trend is observed to a significantly lesser extent in the technical and control groups. This is the same time frame published by Hillenweck et al. for the maximum observed cumulative excretion of chlorothalnil metabolites in rats.[13] Excretion was measured using radiolabeled (^{14}C) chlorothalonil and measuring the radioactivity of collected urine. The amount of metabolite excreted increased logarithmically until leveling out around 50 hours after exposure.[13]

As glutathione depletion is a well-accepted mode of action for chlorothalonil (Davies, 1985), it is reasonable to assume that observed mortality in these trials may be due to depletion of glutathione, which detoxifies this fungicide, as its stored reserves in the bee are exhausted.[16] Once glutathione reserves are exhausted, non-detoxified chlorothalonil can react and modify other thiol-containing coenzymes, enzymes and receptor proteins essential to life, resulting in bee death. However, if mortality was caused by the depletion of water, or pollen reserves necessary to synthesize detoxification enzymes and compounds alone, then statistically it would be expected that observed mortality would continue to increase linearly as more bees deplete stored resources. One possible explanation for this trend is an induction effect on detoxification enzymes as described by Wang et al. in 2010.[9] Future work could focus on investigation of the enzymes involved in chlorothalonil metabolism in honeybees, and investigations of possible induction effects from different chemical agents.

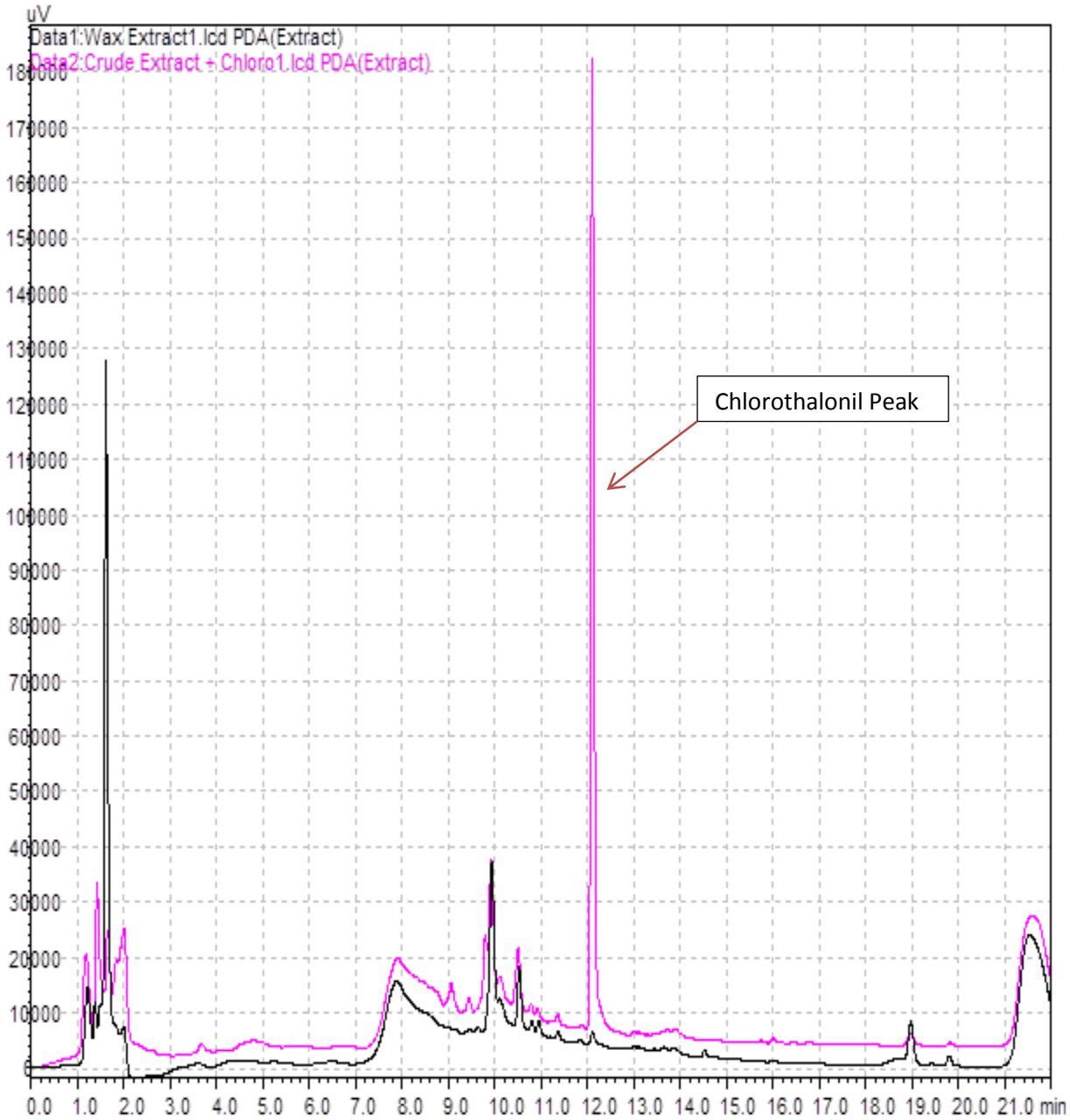
Absorption Spectra Comparisons:

Following the termination of the second trial, all samples (wax, excreta, and regurgitate) were removed from freezer storage and subjected to extraction procedures as described in Biological Methods section, page 13. Samples were then subjected to LCMS UV/Vis spectroscopy as described in the Analytical Methods section, pages 9-10. The following data was obtained from LCMS UV/Vis runs taken using identical time program separation parameters and sample injection volumes. Every effort was made to keep the amount of excreta extracted per sample similar in mass to allow general qualitative comparison. Most displayed data is in the form of UV/Vis spectra monitored at 232nm for maximum detection of the parent chlorothalonil and possible metabolites.[14] Where helpful for comparison, these spectra are displayed as overlays to allow easy comparison between two samples. All displayed data is taken from Trial 2 samples, unless otherwise noted, with the exception of the wax extraction, which was a combination of all wax from both trials. Due to amount of sample available, two distinct samples were taken from each treatment group. Injection volumes for all samples were 2µl.

The first sample consisted of excreta collected 24 hours after initial exposure. A large amount of runny material, presumed to be regurgitate, was also collected at this time. A second sample of excreta was formulated by combining excreta collected from each of the treatment groups at 48 and 72 hours following initial exposure. This combination was necessary to obtain enough excreta for extraction and analysis. Spectra of samples collected at different times are appropriately labeled. All samples from each trial were scanned successively, using identical solvent programs and run times, and every effort was made to keep masses of raw excreta used for extraction similar to allow for some measure of quantitative comparison between spectra in addition to qualitative comparisons with regard to metabolite profile.

As a frame of reference for comparison, a 100ppm chlorothalonil standard was used to spike a sample of extracted excreta from a previous group of bees used to test the excreta collection setup. This spectra is shown in pink in Figure 4.

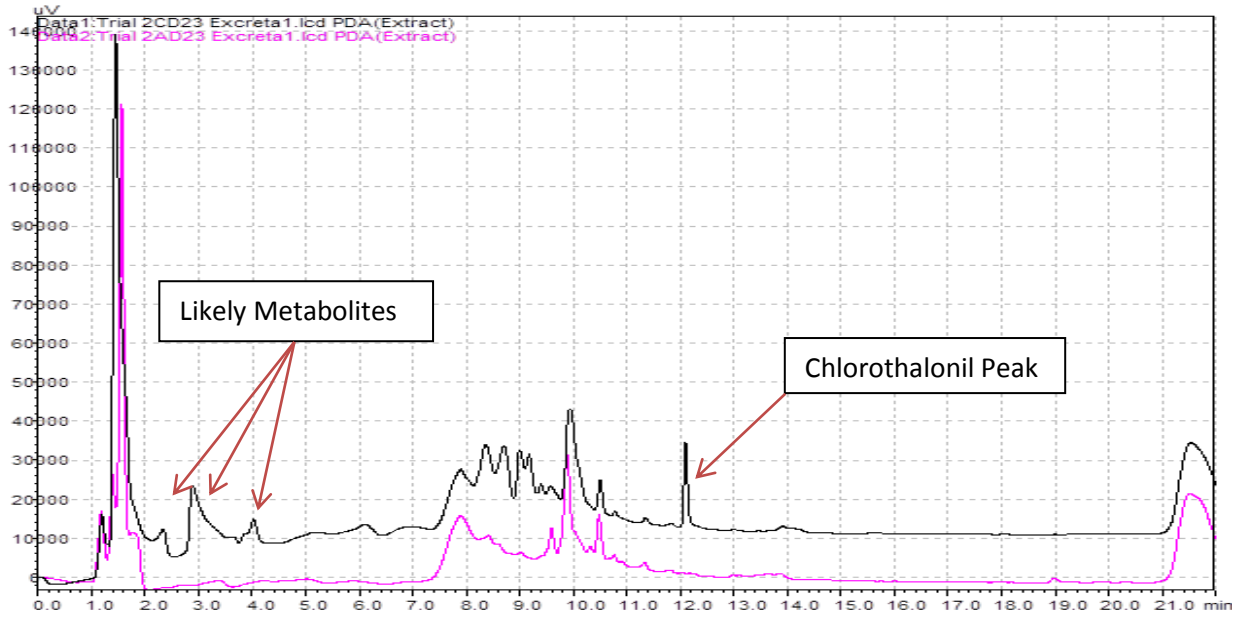
Figure 4: Spectra in pink shows the absorption spectra of honey bee excreta when spiked with a chlorothalonil analytical standard.



This standard allowed the placement of the parent compound at retention time 12.23 minutes into the run of all spectra. Mass spectra of the parent compound in matrix from the formulation treatment group, Trial 2, is shown Figure 12, page 33. Mass spectra shows a 245, 247, and 249 m/z triplet (M^-, M^- + 2, M^- + 4) with relative abundance of the M + 2 peak roughly equal to the M peak, indicative of a compound containing 4 chlorine atoms.[6] These values match data presented by Yamamoto et. al for the negative ion spectra of chlorothalonil.[17] Additionally, UV/Vis spectra shows a maximum at 232nm with a shoulder at 250nm, which matches the spectral values published by Hamersak et al.[14] Since metabolic processes focus on removing non-polar compounds such as chlorothalonil by increasing polarity, all major metabolites should be more polar than the parent compound, and should therefore appear before 12.23 minutes in the absorption spectra.

Initial comparisons were conducted between the control treatment group and the formulation treatment group. The overlay of these absorption spectra is shown in Figure 5.

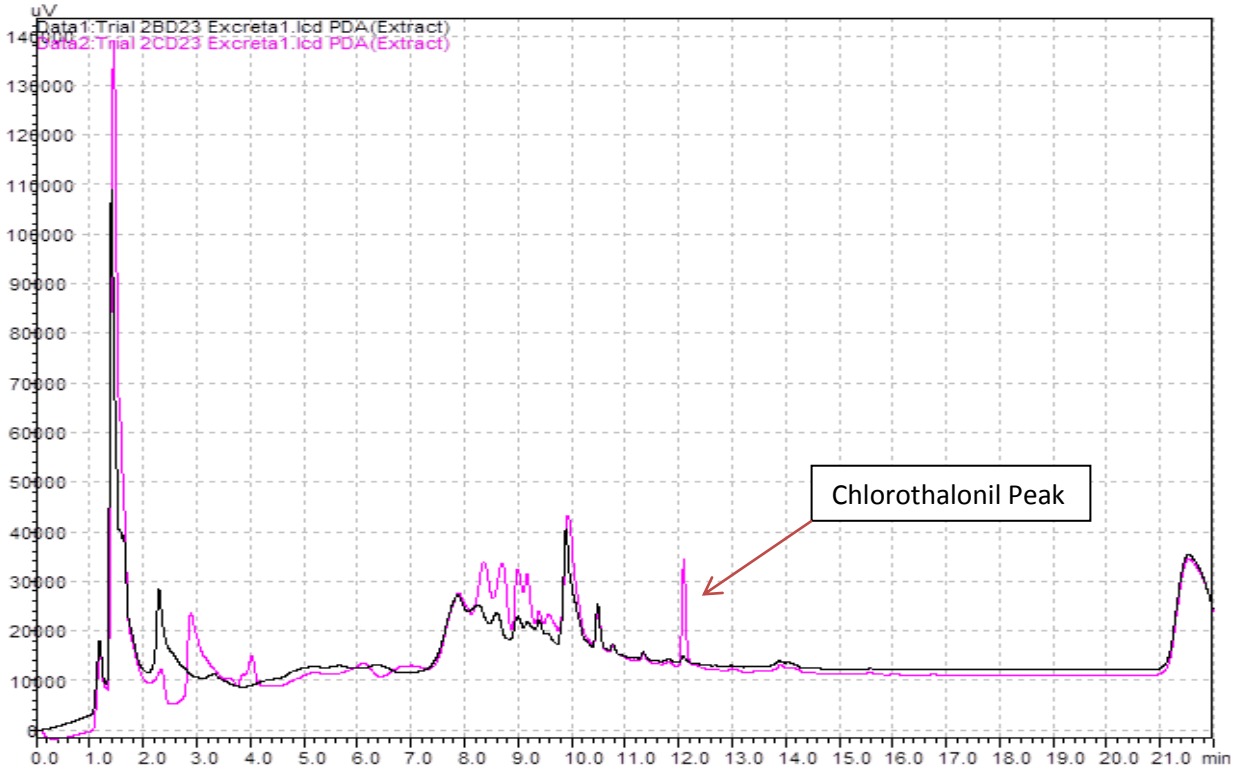
Figure 5: Overlay of the absorption spectra of excreta from the Control and Formulation Treatment Groups, Trial 2, from excreta collected at 2 and 3 days following initial exposure. Spectra in pink represents the control treatment, spectra in black represents the formulation treatment group.



A peak at retention time 12.23 minutes confirmed the presence of chlorothalonil in the formulation treatment group. Lack of this peak showed the absence of chlorothalonil in the control treatment group. In addition, a cluster of three polar compounds located at retention time 2.25, 3, and 4 minutes were observed in the formulation treatment group that were not present in the control treatment. These compounds are likely metabolites of chlorothalonil and future work should focus on their characterization and identification. In a similar comparison, the extraction solvent was subjected to LCMS UV/Vis analysis. Figure 15 in Appendix A, page 39 shows the overlay of the extraction solvent spectra with the control treatment, and confirms that the peaks present at retention time 2.25, 3, 4, and 12.23 minutes are components of the excreta samples, not the extraction solvent.

Once the presence of chlorothalonil and likely metabolites was confirmed, comparisons between technical and formulation treatment groups were begun. Figure 6 highlights the comparison between the metabolite profile of the technical and formulation treatment groups.

Figure 6: Overlay of the absorption spectra of excreta from the Technical and Formulation Treatment Groups, Trial 2, from excreta collected at 2 and 3 days following initial exposure. Spectra in pink represents the formulation treatment group, spectra in black represents the technical treatment group.



The black line shows the spectra of the technical treatment group, while the pink line shows the spectra of the formulation treatment group. While the spectra are similar, there are substantial differences. The peak at retention time 12.23 minutes is significantly larger in the formulation spectra, representing a higher concentration of the parent compound despite equivalent doses. Similarly, the peak at 2.25 minutes is significantly larger in the technical spectrum than the formulation spectrum, while the 3 and 4 minute retention time peaks are present in the formulation spectra and absent from the technical spectra. Since the solvent profile used for the run goes from more polar to less polar, this indicates that the technical treatment group had a higher concentration of more polar (and therefore more highly metabolized) compounds than did the formulation treatment group.

There are possible explanations for these observed differences. One is that in the formulation treatment, chlorothalonil is present at higher concentrations, resulting in depletion of detoxification compounds, causing gradual shift to less highly metabolized compounds. However, concentrations were reasonably well controlled, and the average difference in dose between the technical and formulation treatments does not seem large enough to account for such a dramatic shift. To confirm this, a run of both technical and formulation treatments should have been made. This method will be used in future work.

A more likely explanation is that formulation additives increase movement of parent chlorothalonil to its sites of toxic action, increasing bee mortality and thereby decreasing opportunity for detoxification to polar, excretable metabolites. Similarly, the formulated compound, aided by surfactant additives, penetrates the gut lining and spreads more readily through the bee, resulting in metabolism occurring outside of the normally available gut tissues, causing changes in end products.

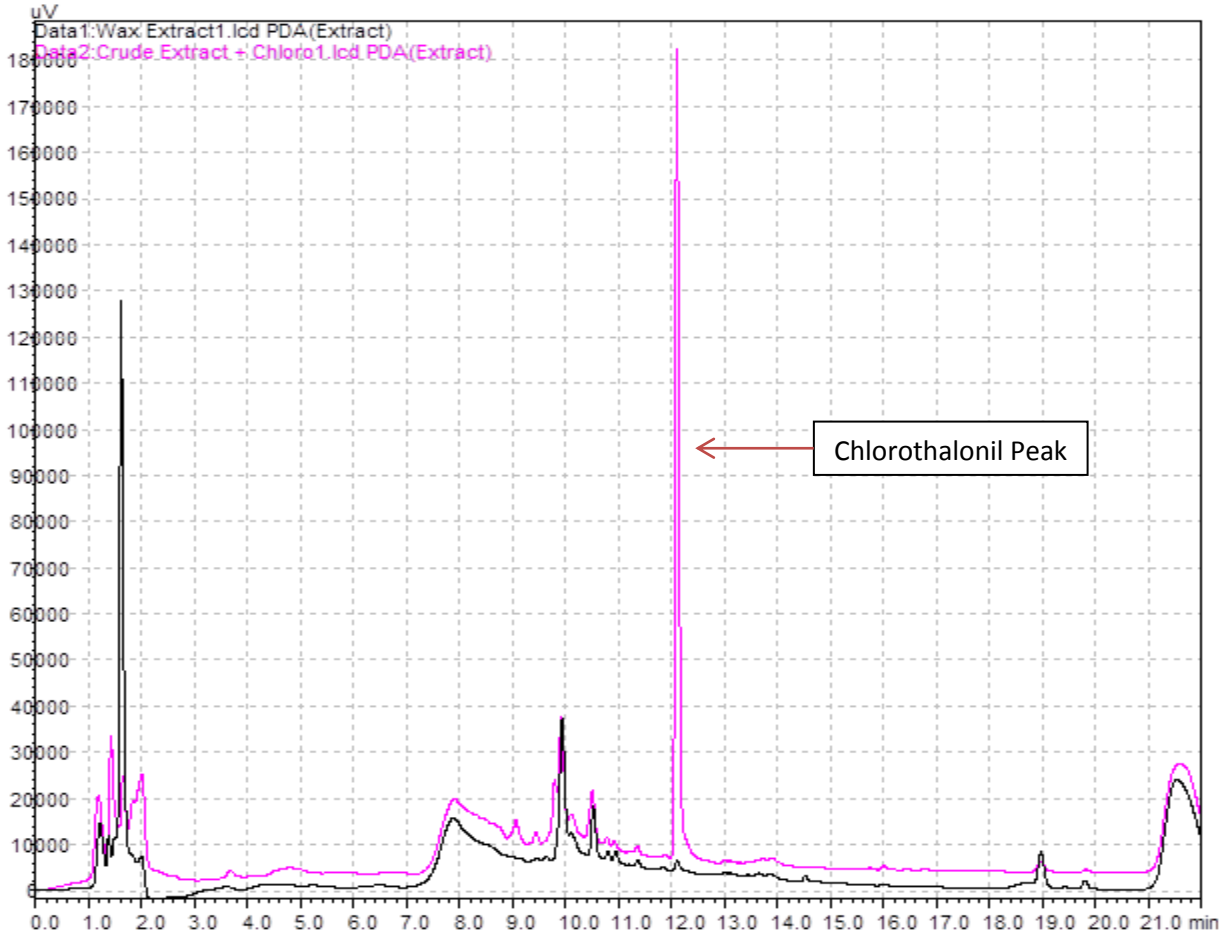
Another possible explanation, based on excreta observations outlined in the Mortality Data section, page 18, involves the role of formulation additives and dehydration. If additives play a role in increasing excreta production, as supported by previously mentioned observations, it is possible that the compound moves more rapidly through the body, allowing less time for full metabolism. Future work should investigate these possibilities to gain more insight into the effects of formulation additives on metabolic processes.

Another interesting hypothesis is if chlorothalonil or its metabolites were being added to wax as a mechanism for detoxification and excretion. Though Mullin et al. have reported chlorothalonil present at high concentrations in beeswax, it is unclear whether the chemical is

deposited from contact of bees with the wax, or if chlorothalonil enters the wax via a metabolic process.[1] This is question is especially significant in light of recent work published by vanEngelsdorp et al. in 2009.[4] Colonies were transplanted into hives using combs from colonies that had recently exhibited CCD symptoms. Combs were subjected to various treatment methods including irradiation and acetic acid fumigation to destroy pathogens as compared to control hives established with combs from healthy colonies. Incidence of entombed pollen was significantly lower in the control group than in any of the treatment groups (11% for the control group versus 53-59% for the treatment groups) suggesting a correlation between comb type and entombed pollen.[4] Since treatments that would have destroyed pathogens were used and found to be ineffective, one possible hypothesis is that a contaminant in the wax of the combs, such as stored pesticides, may play a role in the observed occurrence of entombed pollen.

With this hypothesis in mind, wax chips were collected during all trials from all treatment groups. At the time of analysis, only enough wax for one sample was available by combining all collected wax samples across all treatment groups. Figure 7 shows the spectra of the extracted wax sample in black and the spectra of an excreta sample spiked with chlorothalonil in pink.

Figure 7: Overlay of the absorption spectra of wax collected from all treatment groups from Trials 1 and 2, and a spectra with a spiked chlorothalonil standard. Spectra in black represents wax extract sample, spectra in pink represents an initial excreta extract (no treatment) with a spiked chlorothalonil standard.



The alignment of the peaks at retention time 12.23 minutes demonstrates the presence of chlorothalonil in the collected wax samples. This was confirmed with UV/Vis spectra showing a maxima at 232nm with a shoulder at 250nm, which is in agreement with the Hamersak et al. published values.[14] Since wax chips were collected from outside of containment cages, this data demonstrates that chlorothalonil enters beeswax during wax production, which suggests wax production may be a pathway in a detoxification process. It is also possible that the observed chlorothalonil is a result of pesticide absorption in the gut, aided by surfactants. Future quantitative work could help answer this question.

Once comparison between the technical and formulation groups was satisfactorily accomplished, comparisons between the treatment groups over time were investigated. Samples of excreta from each treatment group collected at one day after initial exposure were analyzed and compared to samples collected from each treatment group at two and three days after initial exposure. Figure 8 shows the spectra of these samples from the technical treatment group, while Figure 9 shows the spectra of these samples from the formulation treatment group. For a spectra of the control group excreta samples in the same time frame, see Figure 14, Appendix A, page 38.

Figure 8: Overlay of the absorption spectra of excreta from the Technical Treatment Group, Trial 2, from excreta collected at 24 hours following initial exposure, and 48 and 72 hours following initial exposure. Spectra in brown represents the sample from 24 hours after initial exposure, spectra in black represents the sample from 48 and 72 hours after initial exposure.

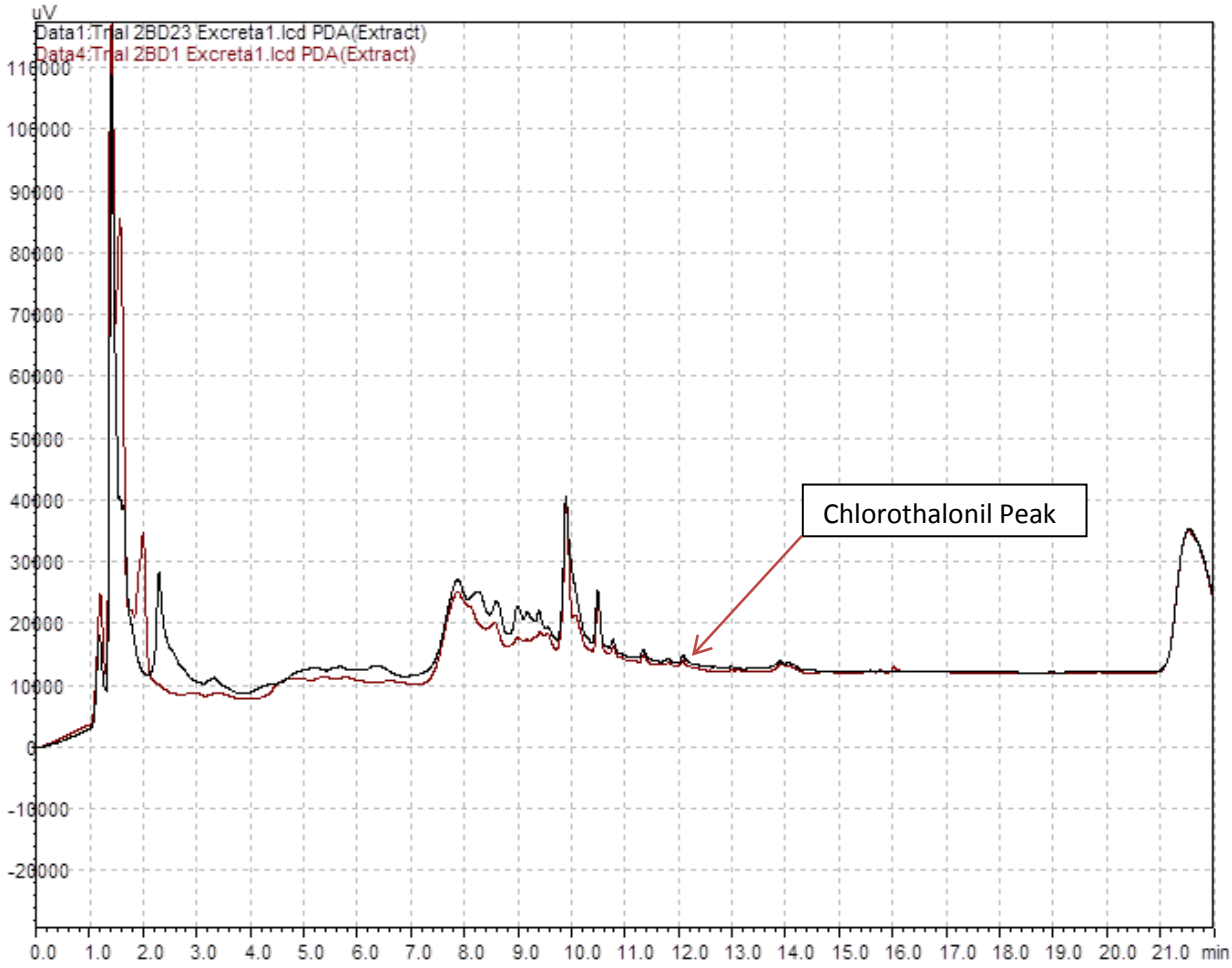
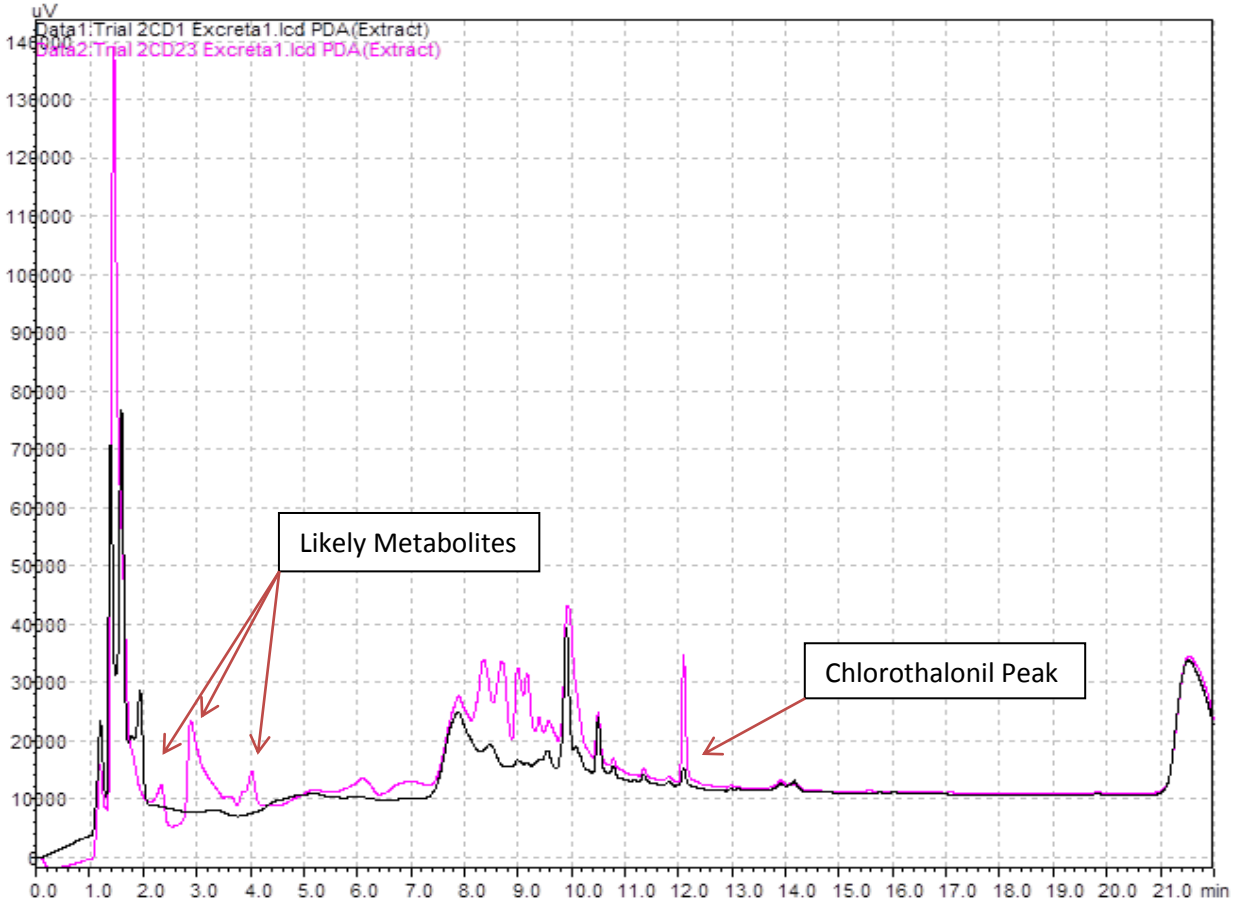


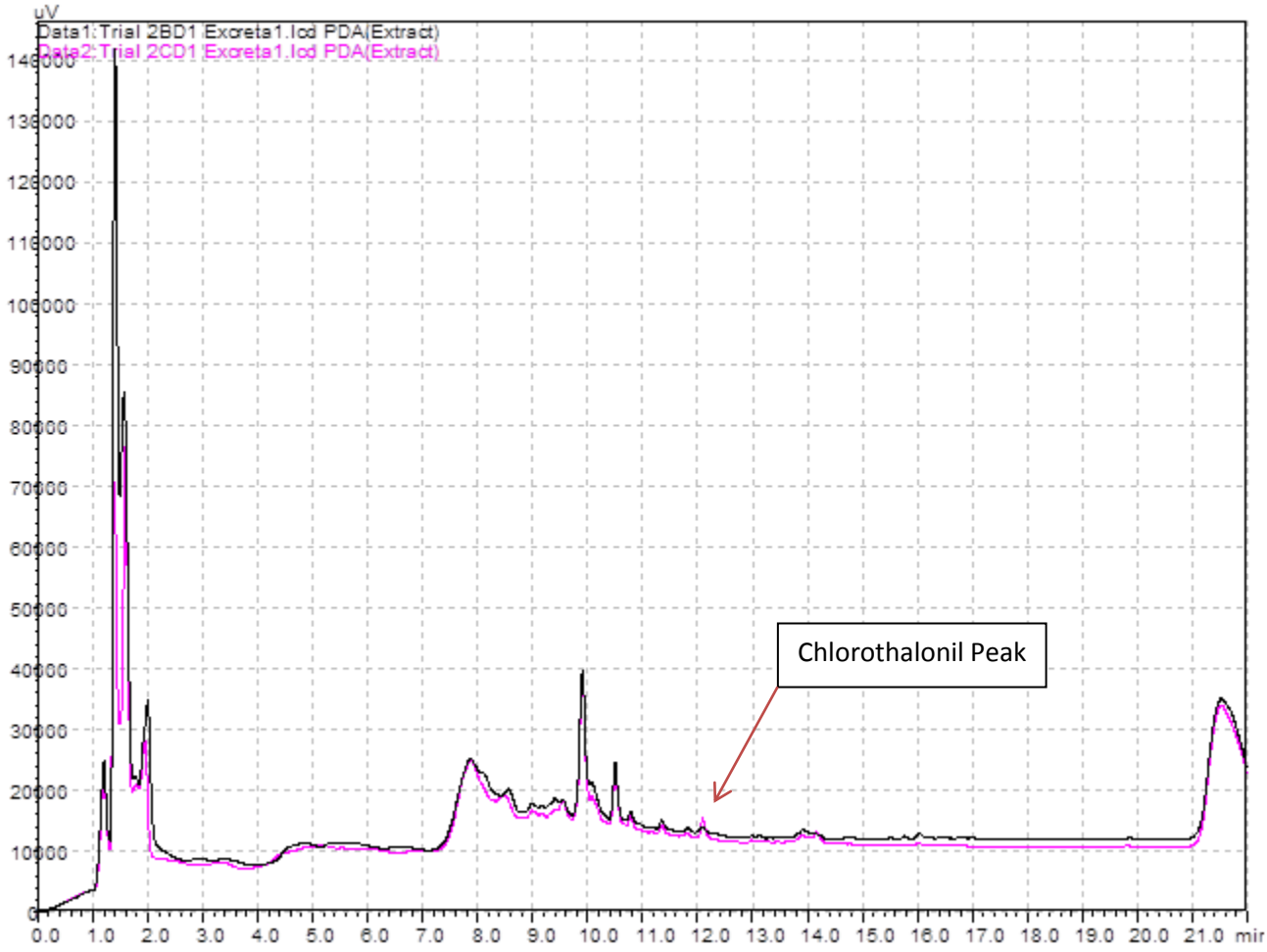
Figure 9: Overlay of the absorption spectra of excreta from the Formulation Treatment Group, Trial 2, from excreta collected at 24 hours following initial exposure, and 48 and 72 hours following initial exposure. Spectra in black represents the sample from 24 hours after initial exposure, spectra in pink represents the sample from 48 and 72 hours after initial exposure.



A qualitative comparison of the spectra in Figure 8 shows little change in the spectra for the technical compound, while Figure 9 shows significant change between the two samples in the formulation treatment group. Based upon this data, then, it seems likely that formulation additives must play some role in this change. Future work should investigate exactly what role such compounds play in altering metabolic processes. It is also interesting to note, as seen from a qualitative comparison between the spectra overlayed in Figure 10, which represent the samples collected at one day after initial exposure for technical and formulation treatment groups, that the metabolite profile at one day after initial exposure looks very similar for both

compounds. This suggest that the observed differences in the formulation and technical metabolite profiles observed in Figure 6 must occur between 24 and 48 hours. Since this time frame corresponds closely with the sharpest rise in mortality observed in both trials, it is reasonable to conclude that this change in metabolite profile results in increased lethality.

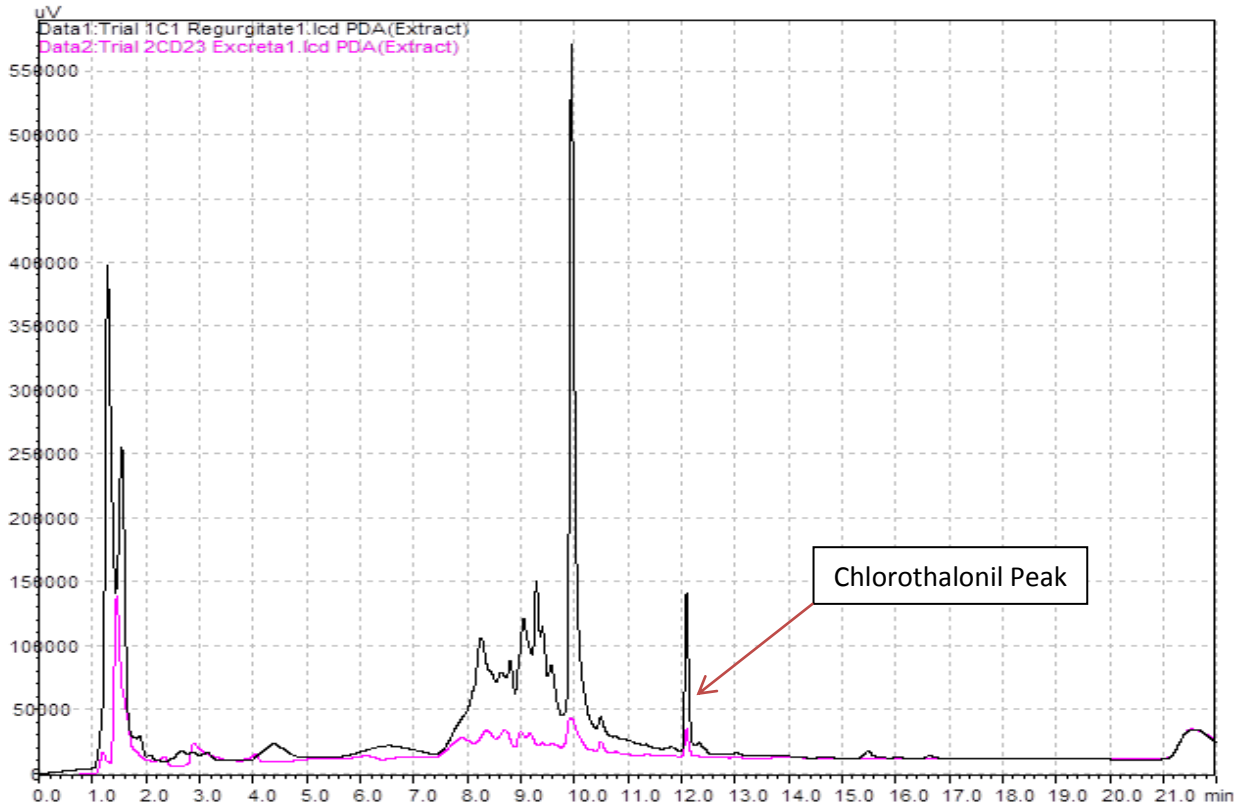
Figure 10: Overlay of the absorption spectra of excreta from the Technical and Formulation Treatment Groups, Trial 2, from excreta collected at 24 hours following initial exposure. Spectra in black represents the sample from technical treatment group, spectra in pink represents the formulation treatment group.



The final sample analyzed in the course of this analysis was collected from the formulation treatment group in Trial 2. The formulation treatment group, as previously mentioned, consistently produced a large amount of runny excreta. Some of this excreta was

produced while the cages were under observation before incubation in a dark growth chamber following the first treatment. Given the short time between sample ingestion and observation, these spots were judged to be regurgitate rather than excreta. Figure 11 shows the spectra of this sample in black overlayed with the spectra of the formulation treatment excreta in pink. A qualitative comparison of the two spectra shows significant differences in chlorothalonil concentration based on the relative sizes of the 12.23 minute peak. Since these regurgitate spots were observed much less frequently in the technical treatment group, it seems likely that formulation additives may play a role in this regurgitation.

Figure 11: Overlay of the absorption spectra from the Formulation Treatment Group, Trial 2, from regurgitate collected at 24 hours following initial exposure, and excreta collected at 48 and 72 hours following initial exposure. Spectra in black represents the regurgitate sample from 24 hours after initial exposure, spectra in pink represents the excreta sample from 48 and 72 hours after initial exposure.



Mass Spectra:

Though the focus of this work was not the quantitative identification of compounds, two mass spectra are presented as confirmation of the presence of parent chlorothalonil and the possible presence of a glutathione conjugate to guide any future work in this area. Figure 12 shows the parent chlorothalonil peak in matrix from the formulation treatment group in Trial 2, chosen because this treatment group had the highest concentration of this metabolite among the excreta samples. The 245, 247, 249 m/z triplet is distinctive for chlorothalonil, and matches values given by Yamamoto et al.[17] The primary ion arises from nucleophilic substitution of water at an ortho position carbon to an activating cyano group and elimination of HCl ($[M+H_2O-HCl]^-$).[8] The 247 m/z peak arises from the presence of chlorine atoms, and represents the presence of the ^{37}Cl isotope with a 25% natural abundance. The greater the relative abundance of the 247 m/z peak in comparison to the 245 m/z peak, the more highly chlorinated the compound. In the case of chlorothalonil, the two relative abundances are almost equal, suggesting a compound with 4 chlorine atoms.[17]

Though the relative abundance ratio of 245/247 in Figure 12 is not 1:1, the identity of the parent is confirmed by UV/Vis spectra which show a maxima at 232nm with a shoulder at 250nm, which match spectral values published by Hamersak et al.[14] It is possible that the discrepancy arises from formation of the hydroxyl-metabolite (4-hydroxy-2,5,6-trichloroisophthalonitrile) in the detector. Similar discrepancies are noted by Yamamoto et al.[17] Figure 12 also gives some idea of the difficulty in isolating one compound for mass spec analysis in this work. While methods for refining the sample are available, use of such methods could have removed possible metabolites, and so crude sample extracts were used, following procedures laid out in the Biological Methods section, page 13. Future work focusing on

characterization and identification of metabolites should make use of additional purification steps.

Figure 12: Mass Spectra of the parent compound primary metabolite in matrix from Trial 2, Formulation Treatment samples, occurs at 12.23 minutes into run.

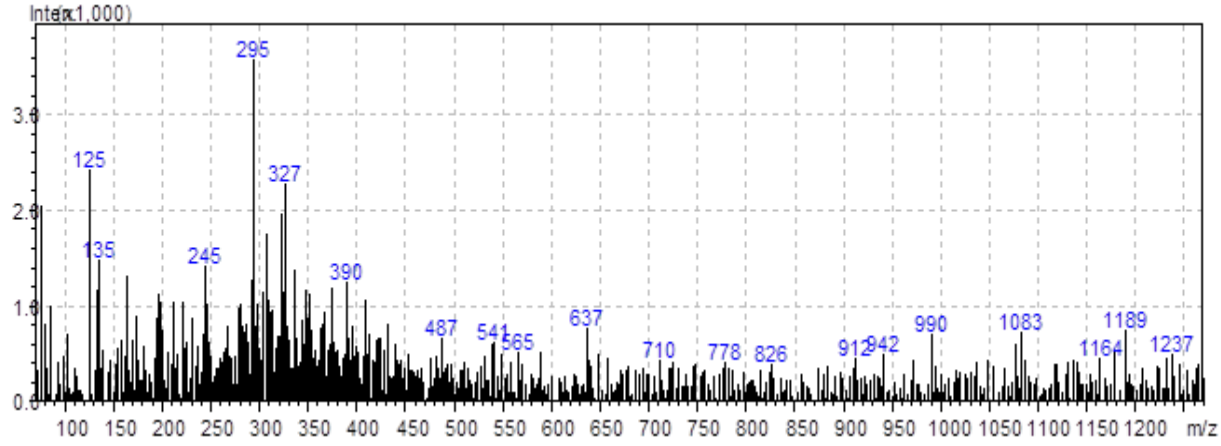
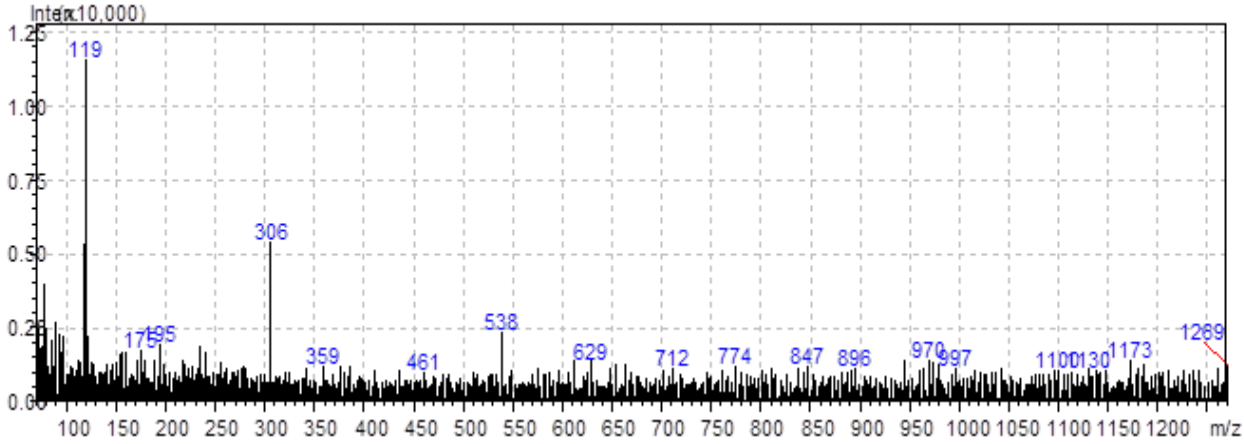


Figure 13 displays the mass spectra of the peak at retention time 2.23 minutes in a sample taken from the formulation treatment group, Trial 2. The primary m/z value of 538 m/z is in agreement with values published by Kim et al. for the mono-conjugate of chlorothalonil with glutathione.[10] Also, the 306m/z value corresponds to m/z values obtained during the preliminary phases of this work when glutathione and chlorothalonil were reacted in water for 30 minutes at room temperature to generate conjugate mixtures that were used to develop a solvent system for LCMS UV/Vis analysis. These values also match those reported by Davies while conducting analysis of glutathione conjugates in freshwater fish.[16] While this spectra is by no means definitive, it is presented as a possible point of investigation for future work.

Figure 13: Mass Spectra of possible glutathione conjugate in regurgitate sample from Trial 2, Formulation Treatment group, occurs at 2.23 minutes into run.



For absorption spectra of control treatment group excreta at 1 Day after initial exposure, and 2 and 3 days after initial exposure, see Figure 14 in Appendix A, page 39

For absorption spectra of extraction solvent versus control group excreta, see Figure 15, Appendix A, page 40

Conclusion

Treatment groups of honey bees were orally dosed with similar concentrations of technical and formulation chlorothalonil. The formulation treatment group displayed increased mortality, and a metabolite profile that changed between 24 and 48 hours after initial exposure, as well as increased excreta production. LCMS UV/Vis analysis shows differences in the metabolite profile of the technical and formulation treatment groups, as well as the presence of chlorothalonil in collected wax chips. Given the similarities in concentration of the active ingredient, this data supports the hypotheses that formulation additives have an effect on metabolic transformations. This data also supports the hypothesis that formulation additives may play a role in Colony Collapse Disorder by contributing to hive stress through dehydration. Future work should focus on characterizing the effects of formulation additives, as well as characterizing and identifying metabolites of common pesticidal agents in honey bees.

Works Cited

1) Mullin, C. A.; Frazier, M.; Frazier, J. L.; Ashcraft, S.; Simonds, R.; vanEngelsdorp, D.; Pettis, J. S., High Levels of Miticides and Agrochemicals in North American Apiaries: Implications for Honey Bee Health. *Plos One* **2010,** 5, (3), e9754.

2) Potter, T. L.; Wauchope, R. D.; Culbreath, A. K., Accumulation and decay of chlorothalonil and selected metabolites in surface soil following foliar application to peanuts. *Environmental Science & Technology* **2001.** *35*, 2634-2639.

3) Vincent, P. G.; Sisler, H. D., Mechanism of antifungal action of 2,4,5,6-tetrachloroisophthalonitrile. *Physiologia Plantarum* **1968.** *21*, 1249-1264.

4) vanEngelsdorp, D ; Evans ,J. D.; Leo Donovall, L.; Mullin, C.; Frazier, M.; Frazier, J.; Tarpy, D.; Hayes , J.; Pettis, J. S. ‘‘Entombed Pollen”: A new condition in honey bee colonies associated with increased risk of colony mortality *Journal of Invertebrate Pathology* **2009.** *101*, 147–149.

5) Arvanties, A.; Boerth, D. Modeling of the mechanism of nucleophilic aromatic substitution of fungicide chlorothalonil by glutathione *J. Mol. Model.* **2001.** *7*, 245-256.

6) Silverstein, R.M.; Bassler, G.; Morrill, T. *Spectrometric Identification of Organic Compounds Fifth Edition*; John Wiley and Sons, Inc.: New York, 1991; pp 3-9

7) Silverstein, R.M.; Bassler, G.; Morrill, T. *Spectrometric Identification of Organic Compounds Fifth Edition*; John Wiley and Sons, Inc.: New York, 1991; pp 289-294

8) Chaves, A.; Shea, D.; Danehower, D. Analysis of chlorothalonil and degradation in soil and water by GC/MS and LC/MS *Chemosphere* [online] **2007.** *71,* 629-638. http://www.sciencedirect.com/science?_ob=MImg&_imagekey=B6V74-4RDB8ST-6-8&_cdi=5832&_user=209810&_pii=S0045653507014191&_origin=gateway&_coverDate=03%2F31%2F2008&_sk=999289995&view=c&wchp=dGLzVlz-zSkWA&md5=f4981890e7f95f12c0ab603ae4347504&ie=/sdarticle.pdf (accessed March 27, 2011).

9) Wang, J.; Jiang, Y.; Chen, S.; Xia, X.; Shi, K.; Zhou, Y.; Yu, Y.; Yu, J. The different responses of glutathione-dependent detoxification pathway to fungicide chlorothalonil and carbendazim in tomato leaves *Chemosphere* **2010.** *79,* 958-965.

10) Kim, Y.; Park, K.; Joo, G.; Jeong, E.; Kim, J.; Rhee, I.; Glutathione-Dependent Biotransformation of the Fungicide Chlorothalonil *J. Agric. Food Chem.* **2004.** *52,* 4192-4206.

11) Davies, P. E., The toxicology and metabolism of chlorothalonil in fish.3. metabolism, enzymatics and detoxication in Salmo spp and Galaxias spp. *Aquatic Toxicology* **1985.** *7*, 277-299.

12) Gallagher, E. P.; Canada, A. T.; Digiulio, R. T., The protective role of glutathione in chlorothalonil-induced toxicity to channel catfish. *Aquatic Toxicology* **1992.** *23*, 155-168.

13) Hillenweck, A.; Corpet, D. E.; Killeen, J. C.; Bliss, M.; Cravedi, J. P., Urinary and biliary metabolic patterns of chlorothalonil in germ-free and conventional rats. *Journal Of Agricultural And Food Chemistry* **1999.** *47*, 2898-2903.

14) Hamersak, Z.; Hollosi, M.; Kontrec, D.; Ladesic, B.; Majer, Z.; Sunjic, V. Preparation and Properties of Glutathione Conjugates of 2,4,5,6-Tetrachloro-1,3-dicyanobenzene *Tetrahedron* **1995.** *51*, 2331-2338.

15) *Bravo Weatherstik (Chlorthalonil)*; MSDS No. 1897-45-6 [online]; Syngenta: Greensburo, NC, Dec. 2, 2004. http://www.falmouthmass.us/cranberry/Bravo%20Weather%20Stik%20MSDS.pdf (accessed March 27, 2011).

16) Davies, P. E., The toxicology and metabolism of chlorothalonil in fish.2. glutathione conjugates and protein-binding. *Aquatic Toxicology* **1985.** *7*, 265-275.

17) Yamamoto, A.; Miyamoto, I.; Kitagawa, M.; Moriwaki, H.; Miyakoda, H.; Kawasaki, H.; Arakawa, R. Analysis of chlorothalonil by liquid chromatography/mass spectrometry using negative-ion atmospheric pressure photoionization. *Analytical Sciences* **2009.** *25*, 693-697

Appendix A

Figure 14: Overlay of the absorption spectra of excreta from the Control Treatment Group, Trial 2, from excreta collected at 24 hours following initial exposure, and 48 and 72 hours following initial exposure. Spectra in black represents the sample from 24 hours after initial exposure, spectra in pink represents the sample from 48 and 72 hours after initial exposure.

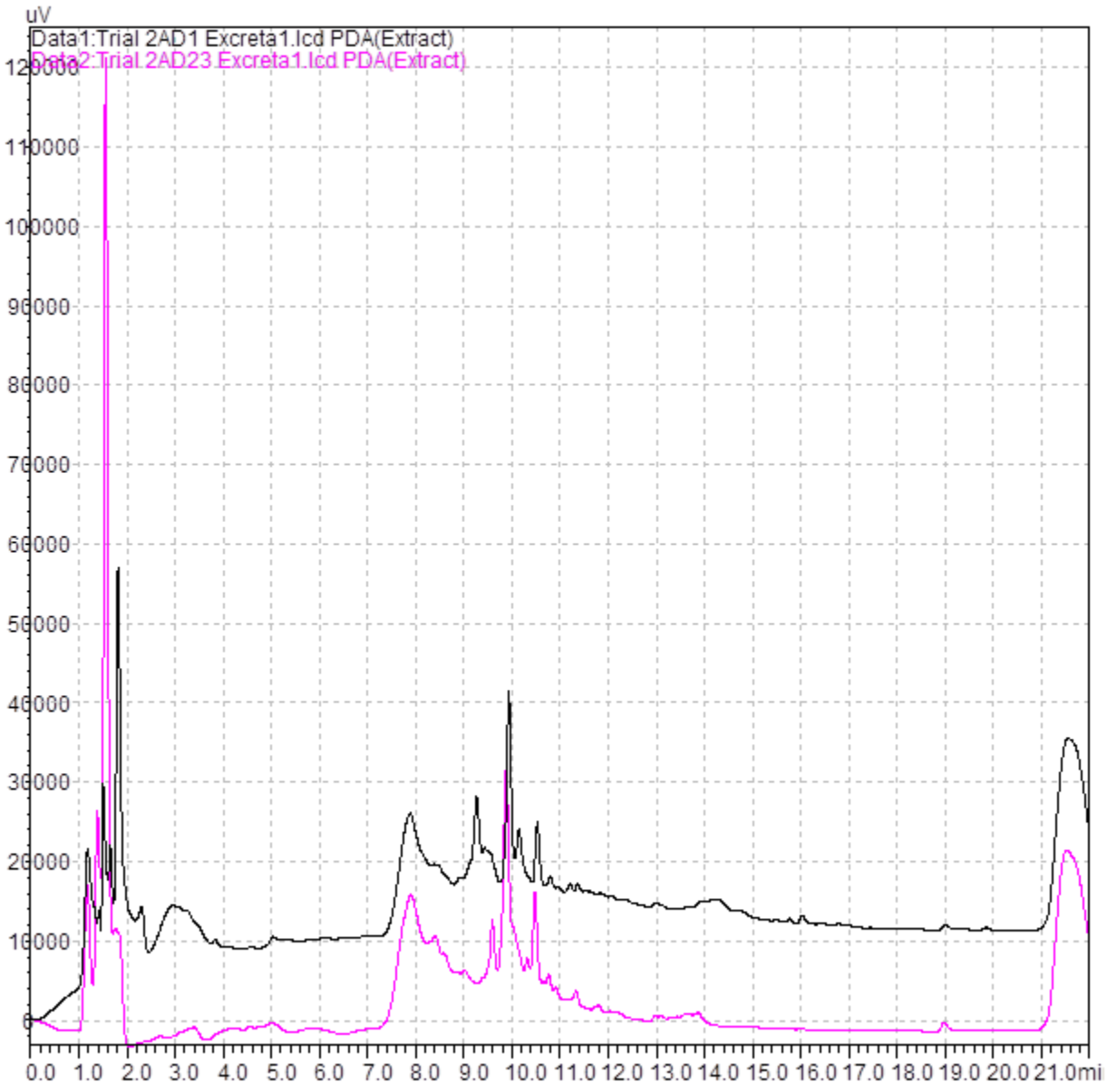
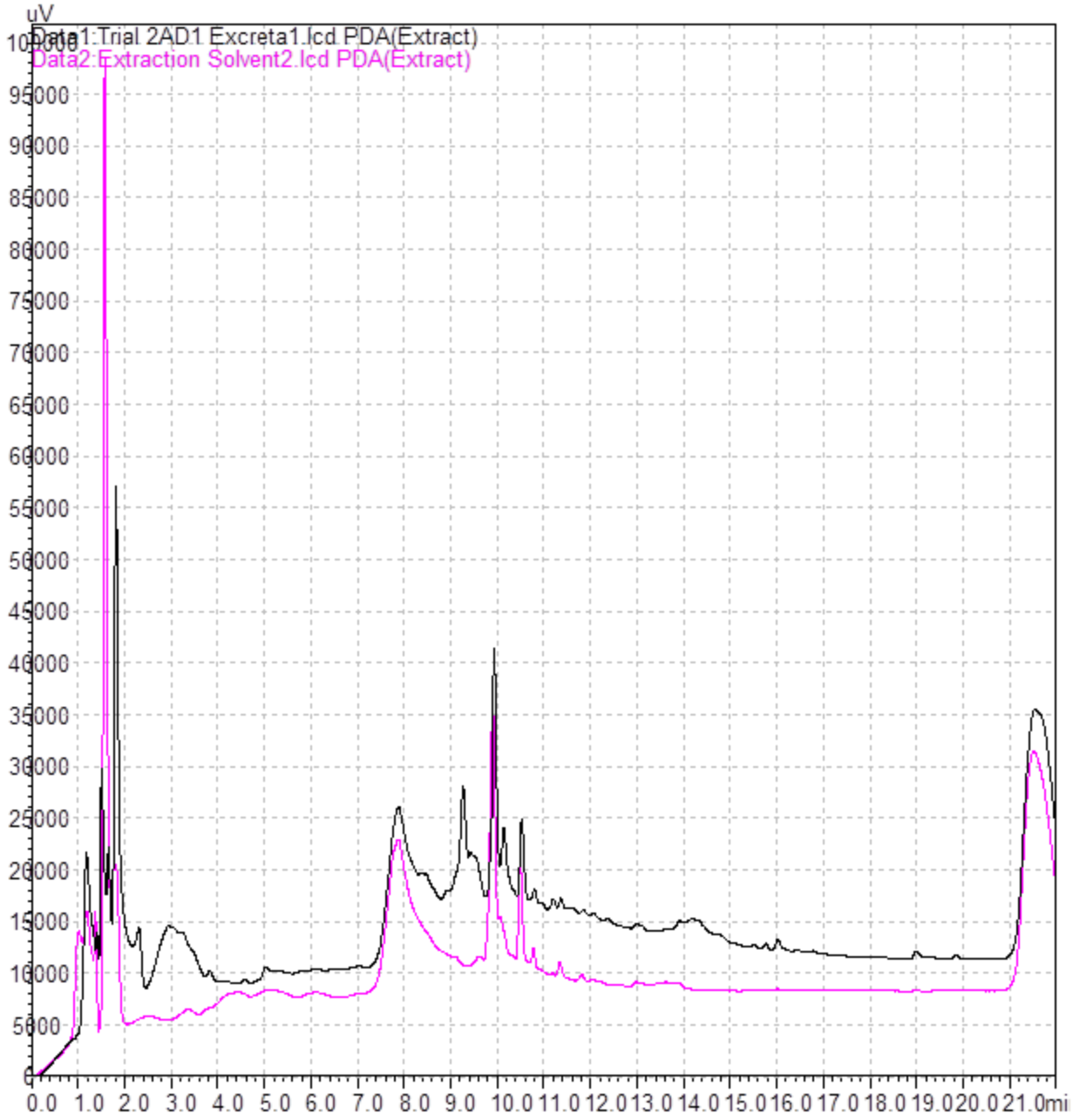


Figure 15: Overlay of the absorption spectra of excreta from the Control Treatment Group, Trial 2, and the extraction solvent system used for all LCMS UV/Vis runs. Spectra in black represents the Control Treatment Group Excreta profile, spectra in pink represents the extraction solvent absorption spectra.



Academic Vita

David Bahn

Permanent Address: 105 S. Main St., York New Salem, PA, 17371

Current Address: 43 Atherton Hall, University Park, PA, 16802

Phone: 717-487-5306 Email: dgb5041@gmail.com

Place of Birth: York, PA Citizenship Status: United States Citizen

Education:

The Pennsylvania State University, University Park, PA, 16802

B.S. in Secondary Education, Certification Area Chemistry

Chemistry Minor, Eberly College of Sciences

Schreyer Scholar

Expected graduation: May 2011

Teaching Experience:

Spring 2011: **Student Teacher (Altoona Area School District)**
15 week student teacher practicum. Designed and implemented lessons for tenth grade general chemistry students, Cooperating Teacher Mrs. Christine Falger

Spring 2010: **Pre-Service Student Teaching (Bald Eagle Area School District)**
8 week middle-level practicum.

Leadership Experience:

Fall 2008-Spring 2010: **Treasurer (Pennsylvania State University Fencing Club)**
Manage club funds (ranging from $5,000-$10,000) and equipment. Organize or conduct inventories of existing equipment, place purchase orders for new equipment.

Fall 2009-Present: **Safety Officer (Pennsylvania State University Fencing Club)**
Maintain Red Cross certifications in CPR and First Aid. Ensure club practices and competitions conform to University safety guidelines. Provide first aid if needed.

June 2007-August 2009: **Junior Assistant Scoutmaster (BSA Troop 149, York New Salem, PA)** Taught Scouting skills including first aid and outdoor survival skills to Scouts ranging in age from 12-18.

Awards/Recognitions:

Eagle Scout, 3/19/2007

ETS Recognition of Excellence for score on Chemistry Praxis

Schreyer Scholar

Professional Organizations:

Student Member, **Pennsylvania State Education Association**

References:

References are available upon request.