# Mid-Infrared Spectroscopy Study of Effects of Neonicotinoids on Forager Honey Bee (*Apis mellifera*) Fat Bodies and Their Connection to Colony Collapse Disorder

Yuzheng Feng[1], Aryan Luthra[1], Kaiwen Ding[1], Yang Yang[1], Jordan Savage[1], Xinrui Wei[1], Roland Moeschter[1], Sachin Ahuja[1], Victor Villegas[1], Bogdana Torbina[1], Dr. Anjuli Ahooja[1]
Dr. Tom Ellis[2], Anna-Maria Boechler[2], Andrew Roberts[3]
[1]*Integrated Science Club, Appleby College, Oakville, ON, Canada*
[2]*Canadian Light Source, University of Saskatchewan, Saskatoon, SK, Canada*
[3]*University of Western Ontario, London, ON, Canada*

## 1. Abstract

This study investigated the negative effects of neonicotinoid pesticides on honey bees in environment surrounding areas of pesticide use. The aim of the experiment is to identify possible contributors to the sudden decrease in honey bee population over the past 60 years, a phenomenon known as the colony collapse disorder. Analysis was performed on three sets of bees: the control group which was not in contact with pesticides, the infected dead group which was a set of bees suspected to have died due to neonicotinoids, and the infected alive group which was suspected to be under the influence of neonicotinoids. After dissecting the bee samples and extracting their fat bodies, the chemical composition and protein structures of the samples were analyzed using Mid-IR beamline at the Canadian Light Source. Results from the spectrographs of bee samples exposed to neonicotinoids demonstrated possible residual pesticide chemicals within fat bodies. Several spectral peaks were also correlated with possible changes in protein secondary structures within fat bodies of neonicotinoid-affected bees. It is likely that the pesticides caused the change in protein assembly structures, which is known to be correlated with disease, paralysis, and death, coinciding with symptoms of Colony Collapse Disorder.

Keywords: neonicotinoids; CCD; honeybee; synchrotron; infrared spectroscopy

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# 2. Introduction

Honeybee colonies are dying at an alarming rate across the world: about one-third of hives collapse every year (Dennis & Kemp, 2016). This is a pattern that has been going on in some areas for over a decade (Pic. 1). For bees and the plants that they pollinate - as well as for beekeepers, farmers, and everyone else who appreciates this marvelous social insect - this is an absolute catastrophe. The prevailing opinion suggests that CCD is linked to multi-factorial causes including pathogen infestation, beekeeping practices, and pesticide exposure in general as was identified in a 2017 CCD study (vanEngelsdorp, et al., 2017). However, a recent finding from Harvard School of Public Health (HSPH) has linked CCD with exposure to neonicotinoids, a group of systematic insecticides that appears to significantly harm honey bee colonies over the harsh winters, in which bees abandon their hives and eventually die. Although this phenomenon is quite well known, the cause is rather elusive. Many claim that it can be attributed to the multitude of causes listed above. However, many beekeepers and farmers believe that CCD should be mostly attributed to pesticides and insecticides sprayed on crops that honey bees pollinate (Aktar, Sengupta, & Chowdhury, 2009).

*Picture 1: An example of a bee colony after colony collapse.*

The most common insecticide sprayed on crops where CCD is observed is neonicotinoids (Pic. 2). Neonicotinoids are insecticides that affect the central nervous system of insects. Many neonicotinoid pesticide products are applied to leaves and are used to treat seeds. They then accumulate in pollen and nectar of treated plants, which may be a source of exposure to pollinators. One of the first neonicotinoids that reached the market was imidacloprid, a common ingredient in garden insecticides. This product can be sprayed on the plant, but is often more effective when applied to the soil. Dinotefuran is another, more highly water-soluble, neonicotinoid that is especially good on sap-feeding insects. (Blacquière, van Gestel, & Smagghe, 2012).

*Picture 2: Neonicotinoids being sprayed on crops*

Although it is said that these insecticides affect the nervous system, there is no understanding of which processes cause this, and how it can be prevented. This study hopes to build on these facts and contribute to information on CCD. In order to further investigate and understand the detrimental effects of neonicotinoid exposure on honey bee populations, we utilized the Mid-IR beamline at the Canadian Light Source in Saskatoon to look at the differences between the composition of the fat bodies found in contaminated bees that are at high risk of CCD and unaffected (organic) bees (through dissection). The fat body is the organ responsible for protein secretion and storage of toxins.

# 3. Theory

As it is commonly known, all light is a form of electromagnetic radiation, and visible light is a small part of this spectrum. Adjacent to the visible spectrum is UV, on the high-energy side, and Infrared (IR), on the low energy side. Mid-IR spectroscopy uses the interactions of IR light with covalent bonds to analyze composition of organic compounds. The range of the electromagnetic spectrum most useful to analyze organic compounds is the 2500-16000 nm wavelength range (Frequency: $1.9\times10^{13}$ Hz to $1.2\times10^{14}$ Hz). The Mid-IR Beamline allows us to use light with wavelength $1.67\times10^{3}$ nm to $1.77\times10^{4}$ nm.

Photons of this energy are not strong enough to ionize atoms or even excite electrons, however, they are energetic enough to induce vibrations in covalent bonds of organic compounds, this is more commonly known as “vibrational excitement.”

Once we recognize that all organic compounds undergo multiple vibrational motions (characteristic of their component atoms and respective hybridization states) and that the bonds in organic compounds act like stiff springs rather than rigid sticks or crystals, it is intuitive that all compounds will absorb IR radiation that correspond with the energy of their characteristic vibrational motions. Since the energies absorbed by a particular compound are unique to it, IR spectroscopy is a useful tool to identify the organic compounds and or functional groups present in a sample. Given enough scans and controlling all variables, one can deduce the presence, amount, sometimes even structure of organic compounds, including proteins, within the sample.

# 4. Experimental Methods

30 forager honey bees were randomly collected from each of the following environments:

- An organic environment that is pesticide-free and the bees are generally healthy. This group is labeled as the Control group.
- A region where bees are dying constantly and were thought to have a high probability of infection during their life span. Collected when dead. This group is labeled as the Infected Dead group.

- A region where bees are dying and were thought to have a high probability of infection. Collected when alive. This group is labeled as the Infected Alive group.

The bees were placed in a sealed container which was then dipped in liquid nitrogen in order to flash freeze the bees. To maintain sample integrity, the bees were then stored in a sterile, airtight, environment and stored at -45 degrees Celsius. They were not removed from storage until it was time to dissect the samples.

The dissection equipment was laid out on the table: scissors, tweezers, scoopula, and scalpel. A desiccator was placed on the table. A dissection microscope was also placed on the table. All the dissection equipment is sterilized with ethanol and Kimtech wipes. The ethanol was first squeezed onto the Kimtech wipes, and the dissection equipment was wiped with the ethanol-wetted Kimtech wipes. A frozen bee was chosen from either the Infected-dead, Infected-alive, or the control group. The bee was placed on the dissection platform. The wings and legs of the bees were cut off by small scissors in order to prevent any possible obstructions during the cutting of the thorax (anatomy shown in Fig. 1). The abdomen of the bee is then separated from the rest of the bee due to the cutting of the thorax. The end of the abdomen is then cut off by about 1-0.5mm to create an opening at the end of the abdomen. An incision was made then from the end to the front of the abdomen on the left or right side of the bee abdomen. Another incision was then made horizontally on the front of the abdomen. A tweezer was used to extend and spread the entire shell of the abdomen part into a sheet. Another tweezer was used to pick up pieces of the fat body from the contents on the sheet of abdomen shell.

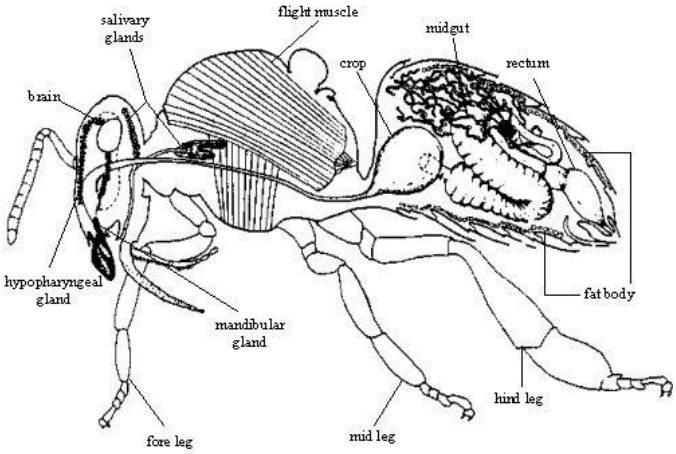
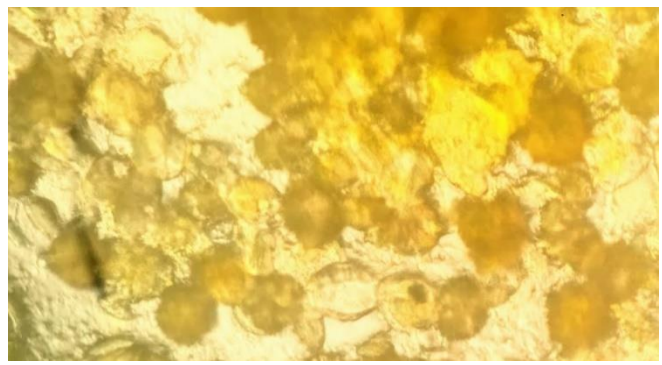



*Figure 1: Anatomical Structure of Honey Bee*

The fat body pieces were placed onto the infrared windows (BaF2). The scoopula was then used to smear the sample of fat body on the infrared window. The window was then placed on the metal window rack to be prepared for transport from the lab to the beamline. Before the transport, the sample is quickly examined under an optical microscope to ensure that it is fat body, and the thickness is appropriate. If the criteria were met (ideal specimen shown in Pic. 3), the samples were then labeled: Infected Dead (herein ID), Infected Alive (herein IA), and Control (herein C).

The samples are then transported to the beamlines. The slide was appropriately placed under the lens and positioned over the sample being tested. An appropriate sampling area (Diameter: 6 µm) was found on the slide using the optical lens. This area was then scanned using IR frequencies (4000-800 Wavenumbers) 255 times in order to reduce background noise. Three possible sampling areas per slide were identified.

This process was repeated for all of the sampling areas on each of the test slides. The spectra were recorded and labeled for analysis. Representative samples were chosen based on how close they were to the "average" spectra in their group. The results were analyzed.



*Picture 3: Ideal Specimen of Fat Body After Extraction*

# 5. Results

The comparison of the Mid-IR spectra of the two groups (Fig. 2) indicates a peak registered by all representative samples of the ID bees group near wave number 1518 $cm^{-1}$. Control group samples, not exposed to neonicotinoids, did not reflect a significant peak on that location of the spectra. This spectra comparison (Fig. 2) also demonstrates a subtle shift in the wave number location of the ID samples' Amide-I protein peak compared to the control group's. Spectra of bee samples exposed to neonicotinoids had Amide-I peaks averaged around wave number 1652 $cm^{-1}$, while control group samples registered their average peak near wave number 1627 $cm^{-1}$. The Amide-I peaks of the ID samples display a smooth shape, mostly with one distinguishable peak, whereas the Amide-I regions of the Control samples contain multiple peaks of varying intensities. The spectra from wave number range 1400~1800 $cm^{-1}$ (Fig. 3) also indicates another unique peak to the ID samples near wave number 1378 $cm^{-1}$.

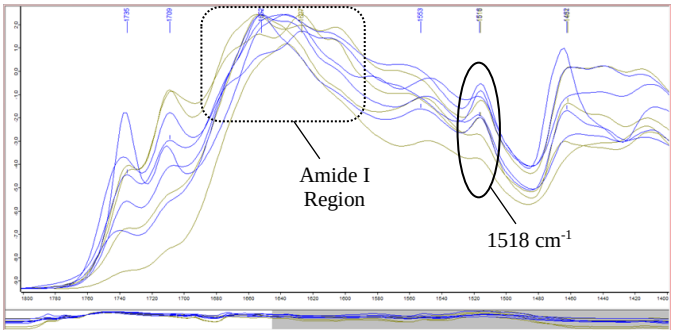



*Figure 2: Mid-Infrared Absorbance Spectrograph of Infected Dead Bees Representative Samples (Blue) and Control Samples (Green) in wave number range 1000~1400 cm$^{-1}$*

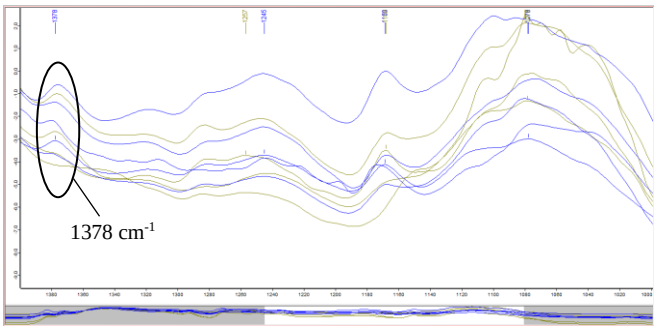



*Figure 3: Mid-Infrared Absorbance Spectrograph of Infected Dead Bees Representative Samples (Blue) and Control Samples (Green) in wave number range 1400~1800 cm$^{-1}$*

The exposure to neonicotinoid pesticides had a less pronounced impact on the spectra of IA samples compared to the impact on the spectra of the ID bees (Fig. 4 Fig. 5). However, 4 out of 5 of the representative samples from the IA group registered an observably intense peak near wave number 1515 cm$^{-1}$. Similar smoothness near the Amide-I region as the spectra of the ID samples was indicated on the IA samples' spectra as well (Fig. 4). Although less discernible, the spectra (Fig. 5) also demonstrates a peak in the spectra of the IA samples near wave number 1377 cm$^{-1}$.

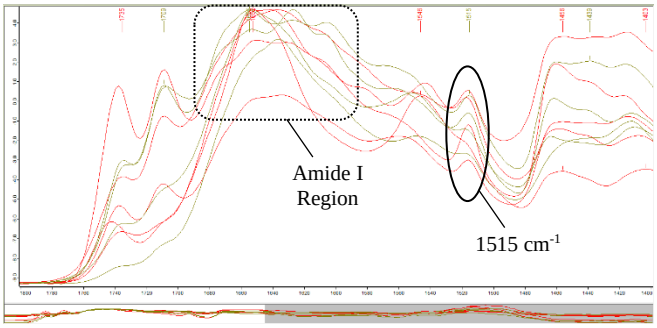



*Figure 4: Mid-Infrared Absorbance Spectrograph of Infected Alive Bees Representative Samples (Red) and Control Samples (Green) in wave number range 1000~1400 cm$^{-1}$*

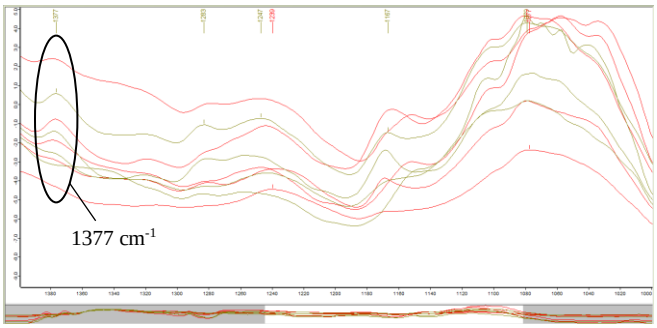



*Figure 5: Mid-Infrared Absorbance Spectrograph of Infected Alive Bees Representative Samples (Red) and Control Samples (Green) in wave number range 1400~1800 cm$^{-1}$*

# 6. Analysis and Discussion

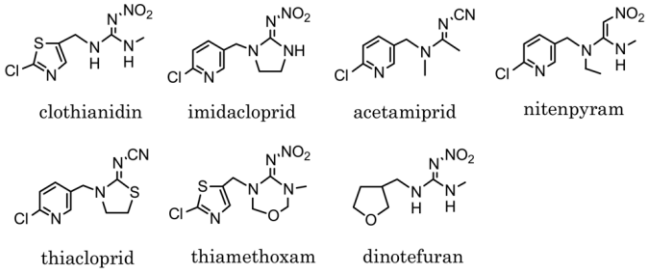



*Figure 6: The Seven Major Types of Neonicotinoids and Their Chemical Structures (Simon-Delso et al., 2014)*

From the chemical structures of the seven major types of neonicotinoids (Fig. 6), it can be identified that 4 out of the 7 types (imidacloprid, acetamiprid, nitenpyram, thiacloprid) contain the functional group 6-chloropyridine ($C_5H_3ClN$) and another 2 out of the 7 types (clothianidin, thiamethoxam) contain the function group 2-clorothiazole ($C_3H_2ClNS$). Due to the lack of pure samples of any of the above neonicotinoids, the analysis of our results was done in the aid of IR spectra of the above functional groups and of smaller constituents of these functional groups.

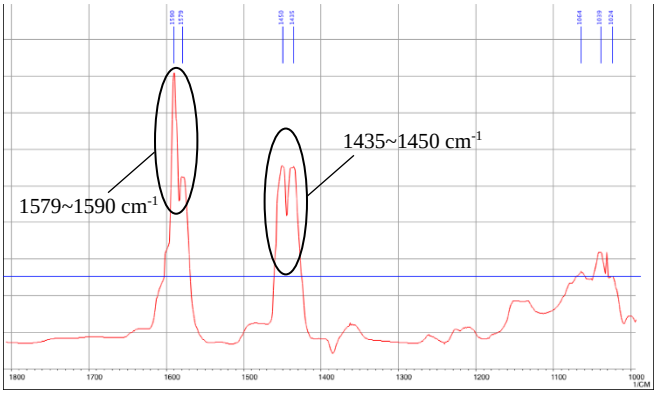



*Figure 7: Partial IR Spectrum of Pyridine (1000~1800 cm$^{-1}$) (NIST, 2016)*

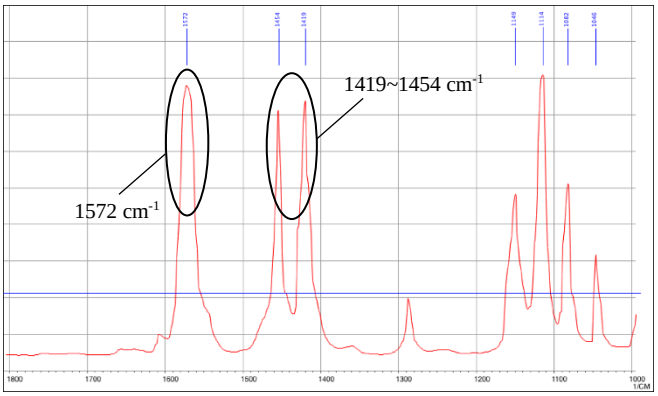



*Figure 8: Partial IR Spectrum of 2-chloropyridine (1000~1800 cm$^{-1}$) (NIST, 2016)*

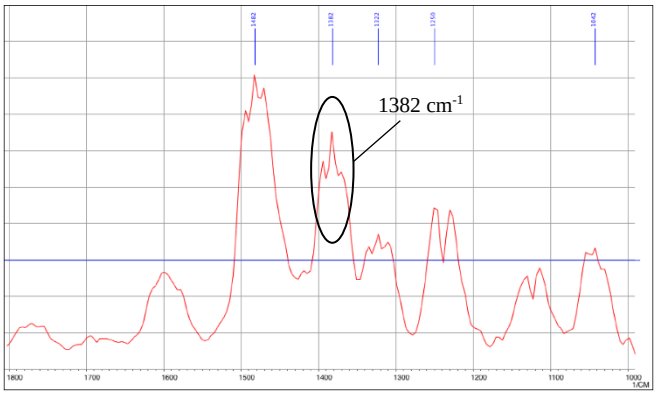



*Figure 9: Partial IR Spectrum of Thiazole (1000~1800 cm$^{-1}$) (NIST, 2016)*

In the IR spectrum of pyridine (Fig. 7), two major peaks are present at wavenumber 1579~1590 cm$^{-1}$ and wavenumber 1435~1450 cm$^{-1}$ region, respectively. A similar pattern is noted in the IR spectrum of 2-chloropyridine (Fig. 8), with two major peaks appearing at wavenumber 1572 cm$^{-1}$ and wavenumber 1419~1454 cm$^{-1}$, respectively. The peaks shifted slightly to the right on the IR spectrum of 2-chloropyridine (Fig. 8) from their locations on the IR spectrum of pyridine (Fig. 7) likely due to hybridization influences from the added chlorine atom.

| | **Peak 1 (cm$^{-1}$)** | **Peak 2 (cm$^{-1}$)** | **Difference (cm$^{-1}$)** |
|---|---|---|---|
| **ID samples** | 1518 | 1378 | 140 |
| **Pyridine** | 1579~1590 | 1435~1450 | 130~155 |
| **2-chloropyridine** | 1572 | 1419~1454 | 118~153 |

*Table 1: Wavenumbers of Major Peaks on IR Spectrographs of ID Samples, Pyridine, and 2-chloropyridine and Their Relative Differences*

The two peaks identified in the results section from the IR spectra of the ID samples (Fig. 2 Fig. 3), at wavenumber 1518 cm$^{-1}$ and 1378 cm$^{-1}$, may not seem related at first to the above-mentioned pyridine peaks. However, further investigation was done through calculating the difference in wave number of the two peaks, which is 140 cm$^{-1}$. This difference was compared to the difference in wavenumber of the two peaks identified on the IR spectrum of pyridine and 2-chloropyridine, respectively. The difference value for pyridine is 130~155 cm$^{-1}$, and the difference value for 2-chloropyridine is 118~153 cm$^{-1}$ (Tab. 1). Although not identical, these three difference values match one another, and 140 cm$^{-1}$ falls into the range for both pyridine and 2-chloropyridine. Since the peaks identified on the IR spectra of the Infected Dead samples are also not far from the pyridine and 2-chloropyridine peaks (less than 100 cm-1), it is highly likely that the ID group's peaks are caused by the presence of pyridine or chloropyridine in the samples. Since the main conditional difference introduced to the ID group is neonicotinoids, and 4 out of 7 types of these pesticides contain chloropyridine, the two peaks on the IR spectra of ID samples are very likely evidence of residual neonicotinoids within the fat body of ID sample bees.

A similar conclusion can be reached with peaks identified on the IR spectra of IA samples, at wavenumber 1515 cm$^{-1}$ and 1377 cm$^{-1}$. The difference in wavenumber of these two peaks is 138 cm$^{-1}$, which is close to the difference value of the ID group and also fits with the difference values of the pyridine and 2-chloropyridine peaks. The IA peaks are very likely evidence of residual neonicotinoids in the system of IA sample bees.

Despite the fact that the majority of the IR absorbance of thiazole occurs in the near-IR region (below 1000 cm$^{-1}$), a relatively significant peak was identified on the IR spectrum (Fig. 9) at wavenumber 1382 cm$^{-1}$. This is almost an exact match to the peak location of the second peak identified on both the ID and IA spectra, at wavenumber 1378 cm$^{-1}$ and 1377 cm$^{-1}$ respectively. The fact that the 1382 cm$^{-1}$ peak is relatively weak on the spectrum of thiazole may explain why the 1378 cm$^{-1}$ and 1377 cm$^{-1}$ peaks are not as apparent on the ID and IA spectra. Although not as convincing as the pyridine evidence, the 1378 cm$^{-1}$ and 1377 cm$^{-1}$ peaks are also possible evidence of the presence of thiazole in the system of sample bees. Since 2 out of the 7 types of neonicotinoids also contain chlorothiazole, these two peaks are may also be evidence of residual neonicotinoids. Neonicotinoids would only remain in the samples bees' fat bodies if the bees failed to breakdown or release the neonicotinoids that were absorbed from the environment. The inability of the honey bees to process such chemicals point to the absence of a mechanism and possible toxicity.

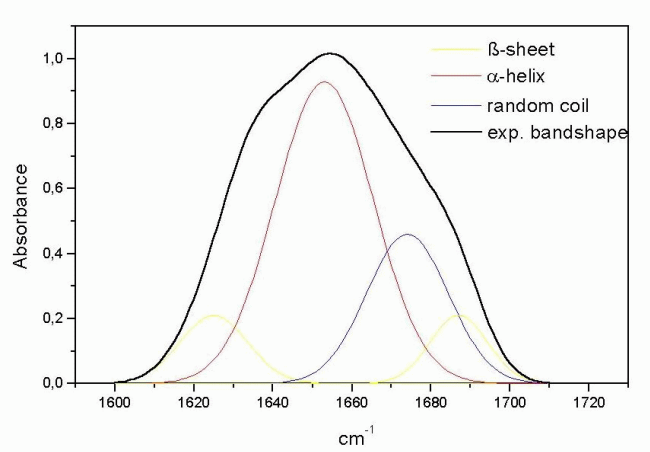



*Figure 9: Deconvoluted IR Spectrum of Amide I Band of a Protein Sample (Jabs, n.d.)*

It is understood that although the difference in wavenumber and similar peak locations point to the likely residual neonicotinoids within sample bees, such analysis may not be the only explanation of our results. Therefore, another possible explanation was explored to explain the ID and IA peaks.

Besides the highly likely presence of residual neonicotinoids within both ID and IA sample bees, our results also point to a possible change in the protein secondary structure of neonicotinoid-affected bees. Before discussing this change in detail, it must be explained that the analysis below was made possible by the pioneering work by Susi & Byler, who proposed a method to deconvolute the Amide-I band of protein samples into the different secondary structures using a Lorentzian shape based algorithm. Their results were verified the results with X-ray crystallography study of the proteins (Susi & Byler, 1986). In the above sample's IR spectrum of proteins (Fig. 9), the peak shapes of different types of protein secondary structure are identified (Jabs, n.d.). Proteins with β-sheet as their most common secondary structure would register two peaks separated by approximately 60 $cm^{-1}$ in wavenumber; however, proteins with α-helix as their secondary structure would register only one peak around wavenumber 1650~1660 $cm^{-1}$. In the above sample spectrum (Fig. 9), the primary secondary structure is α-helix, and the expanded band shape appears smooth with only one peak due to the influence of the intense single peak from the α-helix proteins. The presence of β-sheet and random coil is masked in this Amide I band data. Although no sample spectrum is included with β-sheet as the primary secondary structure, it is evident that the Amide I band of such a sample would have two distinct peaks due to the influence of two intense peaks caused by β-sheet proteins.

As discussed in the results, the relatively smooth shape of the Amide I bands of the ID and IA samples' spectra indicates that the primary secondary structure of proteins is most likely α-helix. The multiple peaks with varying intensities in the Control samples' spectra point to β-sheet as the most likely primary secondary structure of proteins. This major refolding of the protein secondary structures from Control samples to neonicotinoid-affected samples indicates a probable connection to neonicotinoids. Although other random factors could have influenced the shape of the Amide I bands, the relative consistency across all ID and IA samples demonstrate that a general explanation is the most likely, and a change in protein secondary structure is the best justification. Previous studies by Boily et al. (2013) and van der Sluijis et al. (2013) indicate that the neonicotinoids likely cause harm through the inhibition of acetylcholine within the honey bee nervous system. The novel discovery of a change in protein secondary structure in this study could be another pathway through which neonicotinoids cause harm to honey bees. The nature of the study as an IR-based experiment made it impossible for to identify the exact types of proteins being influenced by this change, but further experiments must be performed using other techniques to determine possible proteins that are been affected.

Various recent studies also identify neonicotinoids as the probable culprit behind colony collapse disorder, including the Harvard School of Public Health study by Lu, Warchol & Callahan, and an 18-year distribution study by Woodcock, et al. in England. The Lu study identified impairment of winterization as a major pathway through which neonicotinoids lead to colony collapse disorder (Lu, Warchol, & Callahan, 2014). The British study did not identify any particular pathway of colony collapse disorder; instead, it related distribution of wild bees to that of amounts of neonicotinoid use in oilseed rape and identified the long-term negative impacts of these pesticides on various species of bees (Woodcock, et al., 2016). The results of this study are in agreement with both studies by Lu and Woodcock's teams. This experiment not only indicates the likely presence of residual neonicotinoids in ID and IA samples' fat bodies, it also generates to a novel hypothesis that colony collapse disorder could be the result of a change in the secondary structures of proteins in honey bees.

Further experiments must be done to confirm our results and possibly verify our hypothesis. First and foremost, a more controlled experiment would be performed, with controlled amount of sample bees exposed and the types and concentrations of neonicotinoids the samples are exposed to. This would allow us to perform qualitative analysis of the sample bees' behaviour and also have a better understanding of the peaks we would be expecting from the IR spectra. Control over the type of neonicotinoids would also make it possible to scan the exact pure sample of the pesticide so that its IR spectrum may be used to compare with result spectrographs. Secondly, studies of other possible causes of colony collapse disorder would be carried out, possibly including particulate air pollution, rise in temperature due to Climate Change, and light pollution. Lastly, proper X-ray crystallography studies would

also be ideal, in hopes of identifying the exact proteins involved in the secondary structure change. Although the details of further research are yet to be determined, these studies will certainly help paint a more comprehensive picture of neonicotinoids' relationship to colony collapse disorder.

# 7. Conclusions

By comparing the treatment samples' spectra to the spectra obtained from literature on 2-chloropyrdine and thiazole, it can be said that it is likely that the neonicotinoids are present in the fat bodies of the treated bees. However, they were not present in the control samples, indicating that the insecticide was accumulating within the fat body of the bees. Although these results are likely, it may not be the only explanation of our result.

The size and shape of the Amide-1 peaks in sample spectra and their differences across treatments indicate that there has likely been a change in secondary structures of proteins in the fat bodies of the ID and IA groups. This novel discovery of a change in protein secondary structure from α-helix and β-sheets could be another pathway by which neonicotinoids induce harm to honey bees, as this refolding has been known to cause diseases and paralysis in many organisms.

# 8. Suggestions for Further Research

Although the results of this paper may seem conclusive, it is imperative that our results be corroborated by other experiments. It would be ideal if the experiment could be more controlled. For example, if the bees were grown from eggs by the experimenter and the dose of insecticides was controlled, a more conclusive and quantitative analysis could be performed.

Moving forward, however, it is important to study the effects of these insecticides on bees through multiple techniques. X-ray crystallography could be useful in finding out the exact structures of the proteins being secreted before and after refolding. XRF could also be used to see if the pesticide was causing an increase uptake of toxic elements. IR and XRF spectroscopy could be used to look at the effects of the pesticide on the central nervous system of the bee. It would also be helpful to study the effects of the pesticide on the genes of the bee.

With a more holistic understanding of the effects of neonicotinoids on forager bees, it will be easier to put in place preventative measures, or even, if possible, find a cure. Prevention and cures are also a huge next step in this field of research.

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