

**EXTRACTION OF POTENTIAL CHEMICAL ATTRACTANTS  
FROM *Rudbeckia hirta* INFLORESCENCES**

**A THESIS**

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**BY**

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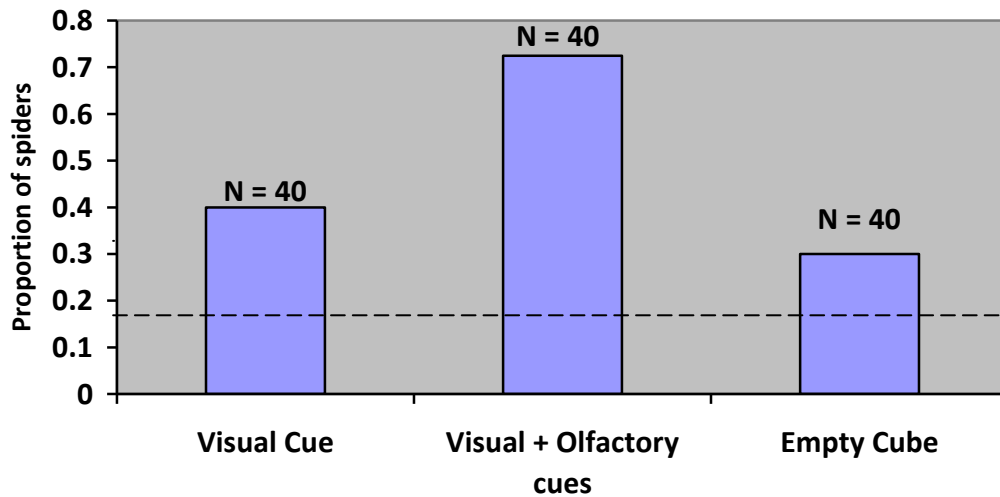
## Chapter 1 Introduction and Background

### 1.1 Purpose of Research

The crab spider *Misumenoides formosipes* has a very interesting mating system. Past research on this spider in our geographic area has shown that sub-adult females commonly forage from the inflorescences of *Rudbeckia hirta*, a particular species of black-eyed Susan. [1] In order for copulation to occur, adult males search their vegetatively complex habitat for receptive females, probably by traveling to the typical foraging locations of these females. This is a very difficult task due to the small size of the male spider in comparison to their vegetatively dense habitat. Previous research supports the hypothesis that the male crab spider use floral chemistry to detect and locate inflorescences of *Rudbeckia hirta*. [1]

In a field study , a polymethyl methacrylate cube with a tight fitting lid mounted on a metal rod was inserted into the ground until it was level with the surrounding vegetation.[1] To test for floral visual cue attractiveness, inflorescences of *R. hirta* were placed in a vial with water and the vial was placed inside of the cube. A male spider was released 25 cm from the front pane of the cube. The release points were approximately distributed 360° around the cube for each trial. To add the potential cue of olfactory attractiveness,these trials were performed with the lid off of the cube. There was also a

control experiment performed where an empty cube was used. Each trial lasted for 2 h. The probability that a spider would end the trials closer to the cube due to random movement was 0.17, based on the net displacement (each spider's starting position to ending position). The results of these trials were that significantly more male spiders ended the trials closer to the inflorescences in all trial categories, but the highest attraction levels occurred when olfactory cues were available. (Fig. 1.1).



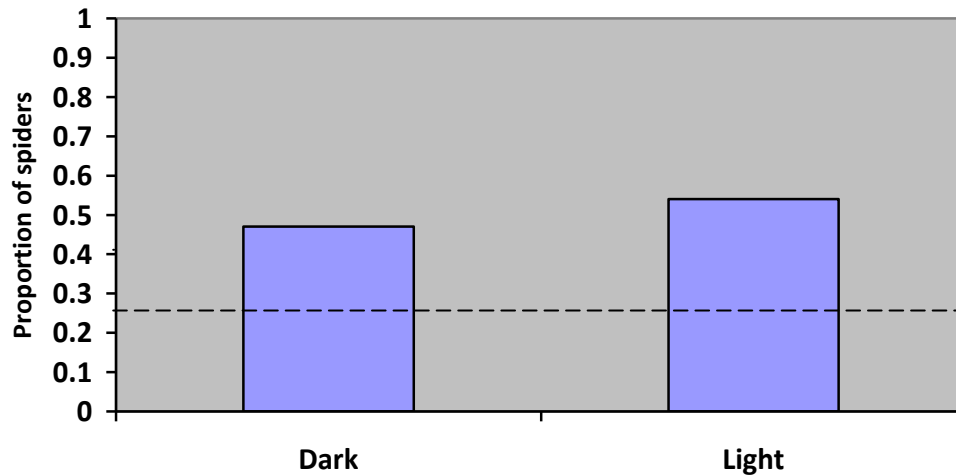
**Figure 1.1:** *M. formosipes* olfactory and visual cues field studies results. Significantly more male spiders ended the trials closer to the inflorescences when olfactory and visual cues were available in comparison to chance. N represents the number of spiders tested in these field trials. The dashed line represents the chance probability. [1]

In laboratory studies, circle arenas were constructed to compare trials carried out in total darkness (eliminating visual cues) to trials carried out with artificial light. The arenas contained four plastic vials that were placed at the corners of a 10 cm x 10 cm

square and one vial was placed in the center. In each trial, three *R. hirta* leaves were placed singly in the corner vials, and one *R. hirta* inflorescence was placed in the remaining corner vial. A male spider was then released onto the center vial and the arena was covered with a glass dome. At the end of 14 h, each spider's location was documented upon first sighting. The proportion of spiders selecting each plant option was analyzed using  $\chi^2$  Goodness of Fit test against the expected probability of ending a trial on a plant substrate (0.25). A  $\chi^2$  Test of Association was used to evaluate differences between dark and lighted trials. In both dark and lighted trials significantly more spiders were found on the inflorescences than on foliage at the end of the trials. However, there was not a significant difference between the lighted and dark trials (Fig. 1.2).

The results from both field and laboratory trials supported the hypothesis that floral cues are used as navigational aids for *M. formosipes* males. In field experiments the males navigated less frequently towards the cube containing flowers when olfactory cues were eliminated. In the laboratory experiments, the male spiders were found on inflorescences rather than the other plant material in both dark and lighted trials. Together, the field and laboratory trials do not dismiss visual cues as a tool of navigation for the males, but they implicate olfactory cues as a significant navigational tool.

The aim of this research is to identify the volatile compounds in inflorescences of *R. hirta* that may be responsible for the olfactory attraction of *M. formosipes* to this plant.



**Figure 1.2:** *M. formosipes* visual cues laboratory results. [1]

## 1.2 Extraction of Volatiles

The literature reports that plant volatiles can be extracted from plant material by various methods. Soxhlet extraction is one of the oldest methods of extraction. It can be used for isolating metabolites from natural materials. It has been used to extract seven crystalline flavonoids from *R. hirta*. [2] In addition to flavonoids, phenolic acids and anthocyanins have been extracted from *R. hirta* using Soxhlet extraction. [3] In this method the solvent is heated to reflux, which is then condensed and allowed to drip onto the plant material. This process is continuously repeated with the recycling of the solvent. It allows for the isolation of analytes of low volatility and thermal stability and high recovery. [4] Yet, it has a long extraction time, large energy and solvent consumption. Another disadvantage is that it has a lowered extraction efficiency caused by the

temperature of the condensed solvent flowing into the thimble is lower than its boiling point. [4] These disadvantages can be partially eliminated by using an automated Soxhlet extractor. This method has been used to determine lipids, and polycyclic aromatic hydrocarbons in natural products. [4]

Accelerated solvent extraction (ASE) is a relatively new extraction technique. This method uses the same principles as the Soxhlet extraction, but it is carried out at an increased pressure, 100-140 atm, and at an elevated temperature, 50-200 °C. [4] This elevated temperature could possibly cause thermal decomposition of compounds. The sample is placed in a stainless steel extraction cell and the cell is pressurized and heated to desired temperature. The sample is extracted statically for a set amount of time. [4] The cell is flushed with solvent after the extract has been removed; this cycle can be repeated. When extraction is complete nitrogen gas is used to move all of the solvent from the cell to a vial for analysis. The increased pressure provides a system in which the temperature can be raised above the boiling point of solvent, yet it keeps the solvent in a liquid state. [4] Under these conditions the solvent has properties that favor extraction. ASE has successfully been used for the extraction of analytes from natural plant products, food, and pharmaceuticals. [4] This method allows for a large quantity of extract in a short amount time, 5-15 min. This is an efficient technique, but its major downfall is the high cost of equipment.

Microwave-assisted extraction (MAE) is based upon the absorption of microwave energy by polar compounds. [4] The energy absorbed is proportional to the dielectric constant of the sample. The higher the dielectric constant, the more energy is absorbed by the sample. This technique is carried out at 150-190 °C. At these temperatures thermally

stable analytes can be isolated. [4] The extraction is carried out in a closed container made of high temperature resistant material. Solvents with low dielectric constants can also be used. In these cases, the sample and solvent is heated, and then the analytes are released into a cooler solvent. This method can be used for the isolation of the analytes that are thermally liable and have low polarity. [4] This technique lowers extraction time and reduces the amount of solvent used.

Steam distillation allows for the extraction of volatile components such as essential oils, some amines, organic acids, and other volatile compounds that are insoluble in water. [4, 5] In this process volatile components are removed by passing steam through the heated mixture followed by condensation of the steam and volatiles. This procedure has substantial energy consumption, and the elevated temperature, around 100 °C, could possibly cause thermal decomposition of compounds. Steam distillation has been used in the evaluation of extracts and oils of tick-repellent plants from Sweden. [5]

Supercritical fluid extraction (SFE) is a technique in which supercritical fluids are used as the extraction solvent. These solvents have high diffusion coefficients, good dissolving power, and low viscosity which allow them to infiltrate plant materials efficiently. Carbon dioxide is commonly used as the solvent with the addition of modifiers in order to enhance the scope of compounds that can be extracted. [4] SFE reduces solvent use and shortens extraction time. In addition, only a small sample size is needed. [4] The technique efficiently extracts compounds with low to medium polarity and high volatility. [4] This technique has been used for the isolation of tocopherols, terpenes, fatty acids, steroids, and triglycerides from plant materials. [4]

Ultrasonic-assisted extraction (USE) allows for the isolation of various types of compounds, both volatile and non-volatile. [4] In this process ultrasound waves, with frequencies that range from 16 kHz to 1 GHz, are the source of energy that assists in the release of analyte from plant material. Cavitation (the generating and collapsing of empty cavities), friction at the interface between solvent and plant material, as well as an increase in diffusion rate is the key to USE. The average time of USE can range from a few to 70 min. The mass of the extract collected is comparable to those collected from dozen of hours of Soxhlet extraction if carried out at the same temperature. This technique can be very time efficient if a solvent is chosen that has a polarity that favors the plant material. In addition, thermal decomposition is not a factor due to the extraction being carried out at room temperature. Many types of compounds can be isolated at once, both volatile and non-volatile. Ultrasonic extraction has been used to extract aroma compounds in aged brandies as well as aqueous alcoholic wood extracts. [6] It has also been employed to extract volatile compounds from citrus flowers and citrus honey. [7] Polysaccharides, cellulose, flavonoids, saturated hydrocarbons, fatty acid esters, and steroids have been extracted from different *Chresta* species using USE. [8]

Cost of equipment, extraction times, as well as solvent use are significant when selecting an extraction technique. Comparison of these factors with the mentioned liquid-solid extraction techniques suggests that ultrasonic-assisted extraction and steam distillation provide low cost of equipment and lowered solvent use. In addition, they are readily available, and they can be carried out in a relatively short amount of time (Table 1.1)

### 1.3 Chromatography of Plant Material

To isolate the various compounds that are extracted from plant material chromatographic methods are utilized. Gas chromatography (GC) with a flame ionization detector (FID) is a popular method of chromatography that is used in the analysis of plant material. In the analysis of steroids and triterpenoids from three *Chresta* species, GC with FID was used. A capillary column made of cross-linked 50% phenyl-methyl-silicone was utilized.

GC with a mass spectrum (MS) detector was used in the identification and quantification of oil and fatty acids content of the fruit of seven *Asteraceae* species. [9] The column was coated with a film of Carbowax 20M (polyethylene glycol).

High pressure liquid chromatography (HPLC) is also a common method used in the chromatographic analysis of compounds found in plant material. HPLC has been used to analyze pentaynene production in *R. hirta*. [10] In these experiments HPLC with a C-18 column with an aqueous acetonitrile mobile phase and detection at 265 nm was used. [10]

Adsorption column chromatography was used in the characterization of flavonoids from *R. hirta*. [2] Glass columns packed with powdered cellulose and



<b>Extraction Method</b>	<b>ASE</b>	<b>MAE</b>	<b>SFE</b>	<b>Soxhlet</b>	<b>Steam dist.</b>	<b>USE</b>
<b>Cost</b>	High	Medium	High	Low	Low	Low
<b>Time</b>	<30 min	<30 min	<60 min	6-48 h	<40 min	<30 min
<b>Solvent (mL)</b>	<100	<40	<10	200-600	<100	<50

ASE= accelerated solvent extraction, MAE=microwave-assisted extraction, SFE=supercritical fluid extraction, Steam dist.=steam distillation, USE=ultrasonic-assisted extraction

**Table 1.1:** Comparison of various liquid-solid extraction techniques used in the analysis of plant metabolites. Steam distillation and ultrasonic-assisted extraction appear to be the most efficient. [4]

polyamide were used. The mobile phases were sequential solvent mixtures of hexane:chloroform:methanol, chloroform:methanol:water, and gradient water:methanol solutions. [2]

GC with FID seems to be useful in the separation and identification of plant volatiles. Standards must be available for comparisons. Therefore, this chromatographic method is most helpful when the compounds or types of compounds being analyzed are known. HPLC is only useful in the analysis of compounds that absorb ultraviolet/visible light. Adsorption column chromatography does not have these limitations if the components can be observed by typical staining methods. Based on the unknown chemical and structural nature of the volatiles that may be extracted in our study, column chromatography was used.

#### **1.4 Olfactometer Bioassays**

Olfactometer bioassays have been used to determine if the compounds isolated from the inflorescences may be potential attractants. [11] There are two main classes of olfactometer bioassays: undiscriminating and discriminating.

Undiscriminating assays do not differentiate between what could be distant and what could be close-range responses to the odor. [11] These assays often have a simple setup and provide a quick method of obtaining statistical data. [11] One type of undiscriminating assay is a horizontal airflow system. In these bioassays the specimen is allowed to maneuver in an airstream that is carrying the odor. Horizontal airflow systems allow the specimen to potentially use the same positive anemotaxis (movement in

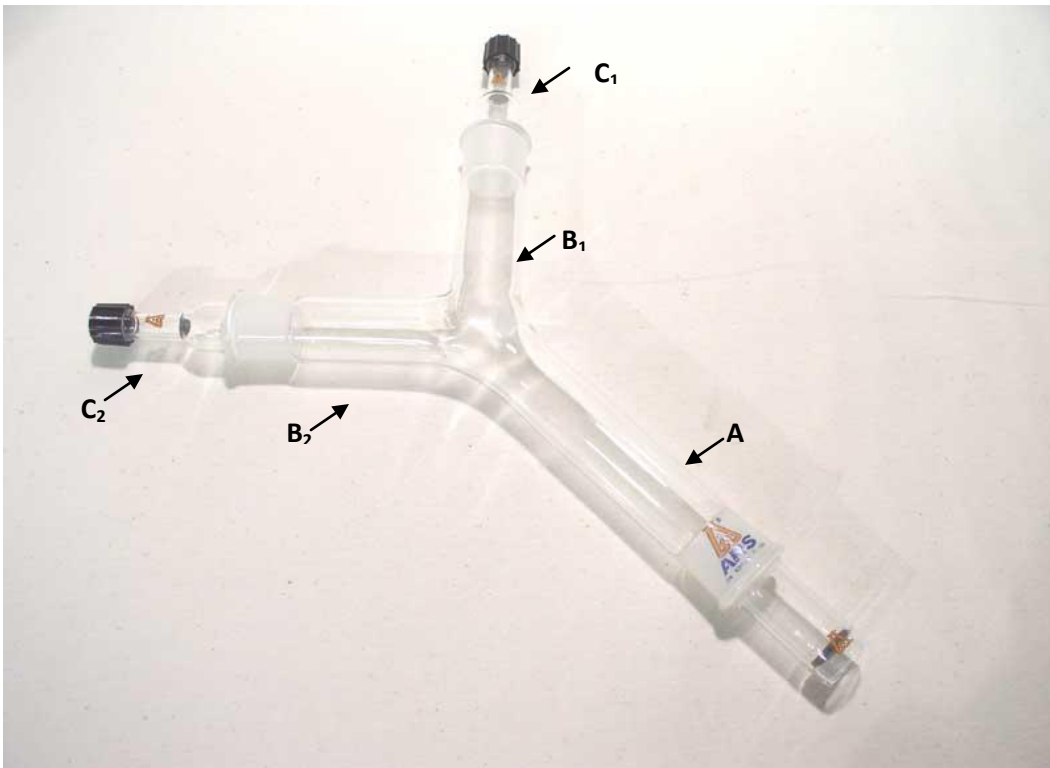
response to wind) for movement toward the sample in the same way it could in the field. [11] In these systems anemotaxis was not explicitly assayed.

The classic y-tube olfactometer is also a horizontal airflow system (Fig. 1.3). [12] There are three major parts of a y-tube olfactometer: an entrance tube, choice tubes, and two sample/control containers. [13] The specimen is introduced into the y-tube through the entrance tube and is allowed to maneuver about the entrance tube for a specified amount of time. The specimen thus has the opportunity to eventually makes its way to a choice tube that is connected to a tube containing either sample or control. In the y-tube is a steep odor gradient at the interface of convergent airstreams. The gradient allows chemotactic (movement toward or away from a chemical stimulus), and chemoklinokinetic (increased or decreased random turning due to a chemical stimulus) responses, which can keep the specimen in one airstream. [11]

Alternative chambers are another type of indiscriminating assay. In most of these systems there is no “intentional” airflow. [11] Therefore, anemotaxis is eliminated but chemotaxis remains as a possibility. These systems include choice chambers, split arenas, and other fragrant and non -fragrant regions in a still-air arena such as the one described on page 3. [11] In other alternative chamber assays there are two adjacent airstreams that ascend vertically through from the floor of a horizontal arena. [11] In these systems anemotaxis is eliminated, and the specimens have the option between the two halves of the arena.

Discriminating bioassays have the same setup as indiscriminating bioassays, yet the odor delivery is varied. For example, in an alternative chamber bioassay the air might be uniformly odorous at any one time, but vary in odor content. [11] An effective

discriminating bioassay has four basic principles. [11] First, the stimulus should only induce one type of response. If more than one type of response is induced, then the bioassay should differentiate between the responses. Secondly, the presentation of the stimulus should be standardized. Thirdly, the responses should be measured at different odor strength. Lastly, the test specimen should be able to respond to the odor in the same manner as it could in the field with adequate space to complete the expected maneuver.



A= entrance tube, B<sub>1</sub> & B<sub>2</sub>=choice tubes, C<sub>1</sub> & C<sub>2</sub>= sample/control container

**Figure 1.3:** Basic y-tube olfactometer bioassay. Many variations exist, but all contain three main parts: entrance tube, choice tubes, and sample/control containers. [12]

### 1.5 Volatiles Found in the *Asteraceae* Family

*R. hirta* is a member of *Asteraceae* (daisy) family. Most research on this family has focused on compounds which have biological and pharmaceutical activities. A growing interest has been in triterpenoids because they have bactericidal, fungicidal, antiviral, cytotoxic, analgesic, anticancer, spermicidal, cardiovascular and antiallergic applications. [8] *Chresta* is one genus of plants in the *Asteraceae* family that has been analyzed due to their richness of triterpenoids. [8] Twenty-six different steroids and triterpenoids were isolated from these plants. [8]

The current research on the compounds that make up *R. hirta* in particular has mainly focused on compounds that are known to be insecticidal or pigments. Polyacetylene derivatives (PAD) have been widely researched due to their insecticidal properties. [10] Various PADs have been isolated from the roots, stems, and inflorescences of *R. hirta*. There are three classes of PADs: polyacetylenes, thiophenes, and thiarubrines and *R. hirta* contains all three. [14] Straight chain PADs are most common. These three classes have been investigated for their insecticidal properties. It was reported that all three exhibited some insecticidal properties either in the light or dark. [14] Also, thiophenes and thiarubrines have been shown to be antibiotic, cytotoxins, and antitumor agents. [15]

Numerous flavonoid pigments have been isolated from the inflorescences of *R. hirta*. [2] Three flavonol glucosides have been shown to act as nectar guide for insects. [16] In addition to flavonoids, essential fatty acids have been extracted from *R. hirta*. [9] Oleic acid, palmitic acid, and linoleic acid were found in high yields in the fruit of *R. hirta*. [9]

Many components of the *Asteraceae* family have been positively identified using carbon-13 and H-1 nuclear magnetic resonance (NMR) spectroscopy. NMR spectroscopy has been used in the identification of flavonoids, dithiacyclohexadienes, and thiophenes. [2-3, 15-17] Ultraviolet (UV) spectroscopy has been used for the detection of flavonoids, thiarubines, thiophenes, and polyacetylenes. [2-3, 14-17] Mass spectrometry has also been used in the identification of flavonoids, dithiacyclohexadienes, and thiophenes. [2, 16-17]

### **1.6 Extraction, Separation, and Bioassay Experiments by Previous Researchers in Our Laboratory**

In preliminary extraction experiments performed in our laboratory steam distillation was carried out for 2 h using approximately 22 g of inflorescences. [18] The inflorescences were pulverized and placed in a steam distillation apparatus with 200 mL of water. Steam distillation was also carried out with macerated inflorescences. The volatiles were extracted from the condensate with dichloromethane and dried over Na<sub>2</sub>SO<sub>4</sub> or molecular sieves. An average of 0.209 g of extract was collected.

Ultrasonic-assisted extraction was also tested as a method of extraction. Solvent solutions of 1:2 hexane:diethyl ether as well as methanol were used. Approximately 10 g samples of *R. hirta* inflorescences were sonicated for 30 min. Ultrasonic-assisted extraction was also carried out using methanol as the solvent.

Ultrasonic extraction provided a larger mass of extract using a smaller mass of inflorescences. In addition, the extraction was carried out in a fraction of the time as well as at room temperature. Thus, the ultrasonic extraction was deemed the most efficient

extraction method. Additionally, there were no added benefits to pulverizing the plant material over macerating the inflorescences. Also, the 1:2 hexane:diethyl ether solvent mixture provided a larger mass of extract than methanol. Therefore, the inflorescences were macerated and sonicated with 1:2 hexane:diethyl ether.

After the volatile compounds are extracted from the inflorescences it is necessary to separate out the components. Previous experiments were performed using a silica flash column with hexane as a mobile phase to facilitate separation. [18] Methanol was used to wash the column after the fractions were collected. Thirty-six 9 mL fractions collected. Each fraction was subjected to thin layer chromatography (TLC) using dichloromethane as the mobile phase. Each TLC plate was treated with a phosphomolybdic acid stain, but only the methanol wash showed any spots. Therefore, it was necessary to develop a more effective solvent system for separation.

Using Erlenmeyer flasks, a y-tube, and Tygon tubing an olfactometer was constructed. [18] The y-tube was covered by a cardboard box. The fractions containing potential attractants were used along with the extraction solvent, hexane, as the control. An individual spider was introduced into the olfactometer. The first preference of the spider was recorded along with the final position of the spider at the end of the 10 min trial. After 10 min elapsed during the first trial the spider was unresponsive; therefore the trial length was increased to 30 min.

Five trials were carried out with the spider's activity monitored for 30 min. These trials provided ambiguous results; fractions containing potential attractants could not be determined. Therefore, the time was increased to 60 min, the airflow was increased, as well as trials were performed in a fluorescent lighted room instead of in the dark. Both

fractions and intact inflorescences were used as potential attractants. Seventeen of these trials were carried out. These trials also provided ambiguous results and a potential attractant could not be determined. Experimental parameters for performing y-tube olfactometer bioassays, which deliver olfactory cues to the spiders, needed to be further explored and developed.

### **1.7 Experimental Approach**

From the review of the literature and the results of past experiments, we concluded that the overall experimental approach to our research would be to use ultrasonic-assisted extraction to facilitate the discharge of volatile compounds from the inflorescences of *R. hirta*. Next, the bulk extract would be separated into fractions that contain similar components using flash column chromatography along with an effective solvent system. The fractions that are collected would then be pooled according to TLC results. Subsequently, the pooled fractions would be tested for attractiveness using a y-tube olfactometer bioassay. The pooled fractions would be analyzed using infrared spectroscopy, NMR spectroscopy, and other analytical techniques to identify the chemical components.



## **Chapter 2**

### **Chromatographic Method and Bioassay Development Method**

Based on the results from previous researchers' separation experiments, it was necessary to develop a solvent system that yields better separation of components. This was accomplished by experimenting with different solvent solutions of varying polarity. Additionally, development of bioassay parameters was determined by performing olfactometric trials with *Drosophila melongaster* and a modified y-tube olfactometer bioassay.

#### **2.1 Initial Chromatography Experiments**

##### **2.1.1 Experimental**

An ultrasonic-assisted extraction was performed for 30 min using 10 g of macerated *R. hirta* inflorescences. The inflorescences were collected from Cooper Woods (Muncie, IN) approximately 1 month prior and were placed in the freezer until the time of extraction. The inflorescences were placed into a 125 mL Erlenmeyer flask along with 60 mL of 1:2 hexane (Fisher Scientific):diethyl ether (Sigma-Aldrich,  $\geq 99.5\%$ ) solution as the solvent. After 30 min the flask was removed and the extract was gravity filtered. The solvent was removed from the filtrate by using a gentle stream of argon. The filtrate of

the extract was reconstituted in 3 mL of dichloromethane (Sigma-Aldrich,  $\geq 99.5\%$ ) and injected into a silica flash chromatography column (Analogix SF25-40g, Sepra Si 50), and washed through with an additional 2 mL of dichloromethane. Dichloromethane was used as the mobile phase, and methanol (Fischer Scientific,  $\geq 99.8\%$ ) was used to wash the column after the fractions were collected. A total of thirty-six 9 mL fractions were collected.

Thin layer chromatography (TLC) was performed on each of the fractions (Bakerflex 2.5 x 7.5 cm, silica gel IB-F) by spotting each TLC plate with 5 drops of the fraction, and then placed into a solvent chamber. Various solvent solutions were tested as to their effectiveness. These included 100% dichloromethane, 100% hexane, 1:20 dichloromethane:hexane (hereafter referred to as 95% hexane), and 1:20 hexane:dichloromethane (hereafter referred to as 95% dichloromethane). Each subsequent plate was viewed under ultraviolet light, both short and long wavelengths, for active spots. Subsequently, each plate was stained with a phosphomolybdic acid (PMA) stain (1:25 PMA: 95% ethanol), and gently heated.

### **2.1.2 Results and Discussion**

The resulting TLC plates from the separation carried out with dichloromethane showed good separation of components except for those in the first two fractions. These components were not separated well near the point of application as well as one heavy spot near/on the solvent line. The resultant TLC plates from using 95% dichloromethane as the mobile phase showed separation for the spots near the application line. When 100% hexane was used as the mobile phase the TLC plates from these experiments

showed that some components did not move at all at the application line, but the spot at the solvent line separated into two. Using 95% hexane as the mobile phase yielded better separation of these particular spots. These results along with the results from the separation experiments discussed in Section 1.7 suggest that if a multiple solvent system was used in the flash chromatographic separation, it would provide for a more effective separation of components.

## **2.2 Flash Chromatography Experiments with Sequential Solvent System**

Again, based on the results described above, a series of solvent systems were tested for the effectiveness in the flash chromatography separation. One hundred percent hexane, 95% hexane, 95% dichloromethane, and 100% dichloromethane were used for the next separation.

### **2.2.1 Experimental**

Ten grams of frozen *R. hirta* inflorescences were subjected to ultrasonic-assisted extraction as stated in Section 2.1.1. Using the series of solvents as the mobile phases, flash column chromatography was carried out using the same procedure stated in Section 2.1.1, except 60 fractions were collected. Ten fractions were collected using hexane, 20 were collected using 95% hexane, 20 were collected using 95% dichloromethane, and 20 were collected using 100% dichloromethane. Subsequently, each fraction was subjected to TLC as stated in Section 2.1.1.

### **2.2.2 Results and Discussion**

The resulting 100% hexane fractions were colorless and their TLC plates did not show any spots. Fractions collected using 95% hexane, 95% dichloromethane, and 100% dichloromethane, were either colored and/or showed spots on the TLC plates.

No components could be detected in the fractions collected with the 100% hexane solvent, and it was not used in future separations. Ninety-five percent hexane, 95% dichloromethane, 100% dichloromethane, and methanol were used in sequence as the mobile phases for flash chromatography in order to facilitate separation of components.

### **2.3 Infrared Spectroscopy of Fractions**

In order to determine the types of compounds extracted from *R. hirta* inflorescences using our methods, Fourier transform infrared (FT-IR) spectra were obtained on pooled fractions.

#### **2.3.1 Experimental**

Infrared spectroscopy was carried out on the fractions from the previous separation discussed in Section 2.2.1 and were pooled together based upon similar  $R_f$  values and/or color. Three drops of the fraction were placed on a KBr salt plate and the solvent was allowed to dry creating a thin film. Infrared spectra were obtained using a Perkin-Elmer Spectrum 100 infrared spectrometer. The number of signal-averaged scans was 10, the resolution was  $4.00\text{ cm}^{-1}$ , and the baseline was manually corrected.

### 2.3.2 Results and Discussion

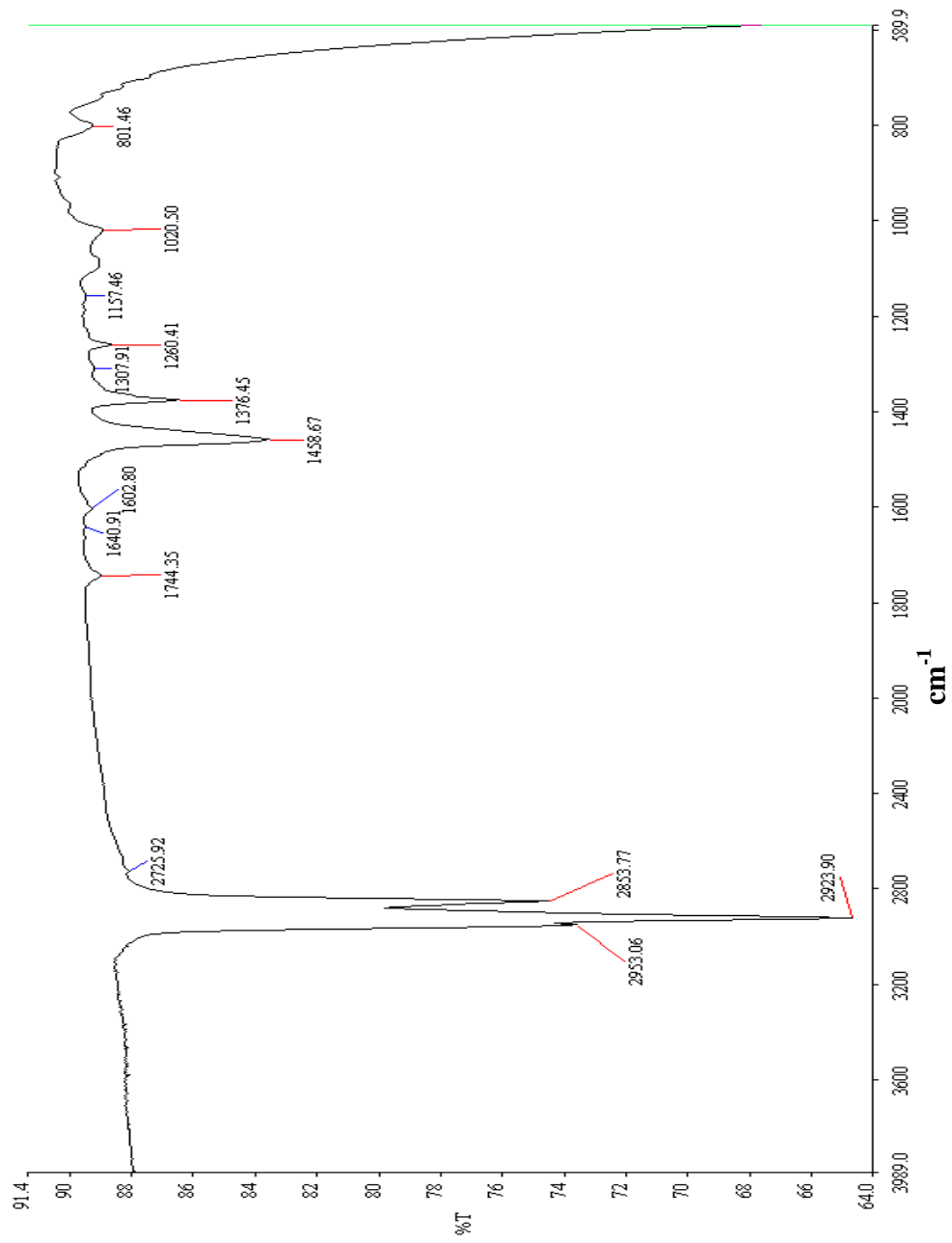
The FT-IR spectrum of the pooled fractions collected with 95% hexane, shown in Figure 2.1, has an absorption at  $1745\text{ cm}^{-1}$  which is consistent with C=O stretch, and since it is at a relatively high frequency we propose corresponds to an unconjugated ester. Additionally, methyl and methylene stretching and bending modes are observed, and thus a long chain fatty acid ester could be a component of these fractions. The spectrum of fraction 45, which was collected with 100% dichloromethane, shown in Figure 2.2, has absorption at  $1735\text{ cm}^{-1}$ , which is indicative of an C=O stretch and the frequency is consistent with a conjugated ester. Figure 2.3 is a spectrum of pooled fractions 51-57 which were collected with 100% dichloromethane, and it has an interesting band at  $2223\text{ cm}^{-1}$  which is consistent with a C≡C stretching absorption. Additionally, there is absorption at  $3008\text{ cm}^{-1}$  which is indicative of a CH stretch of an unsaturated group. Together, these suggest a decomposition product from a thiarubrine (Fig. 2.3a), a compound known to be in the *R. hirta*. [14] This spectrum also has an intense band at  $1745\text{ cm}^{-1}$  which is indicative of ester absorption which has probably been eluted along with the thiarubrine. One should note that the ester frequency is the same as that observed in the spectrum of the 95% hexane pool. However, the relative intensity of that absorption compared to the methylene stretches in Figure 2.3 is much greater than that observed in Figure 2.1. This probably indicates that a long chain alkane probably eluted with the fatty acid ester in the 95% hexane pool.

## 2.4 Bioassay Experiments

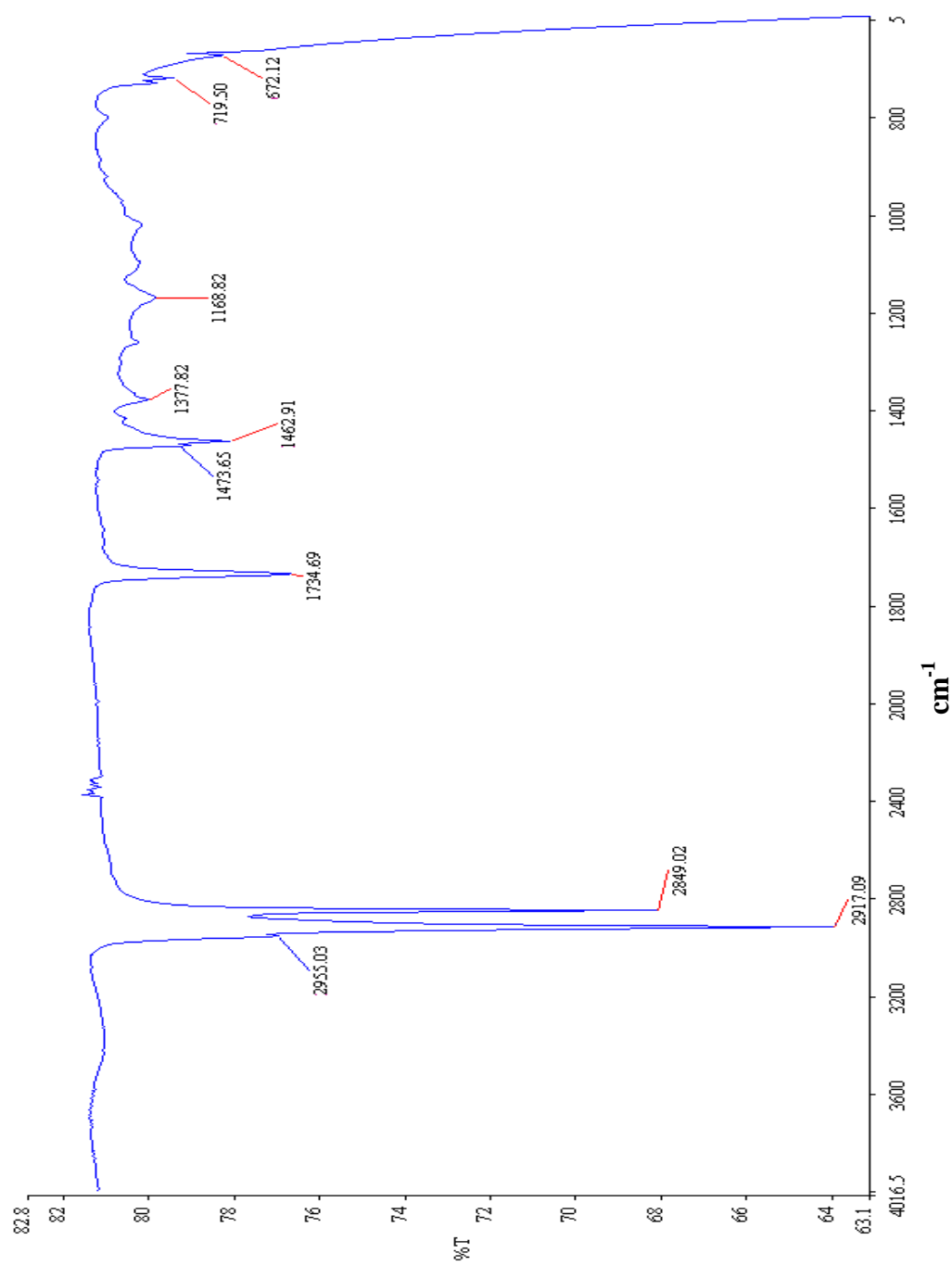
Due to the ambiguity of previous bioassay trials, a more effective bioassay was needed. *M. formosipes* mating season is relatively short; consequently, in order to maximize the number of spider olfactometer trials that could be completed, the newly constructed bioassay was tested with a flightless strain of *Drosophila melongaster* prior to the mating season of the crab spider. These trials were used to determine the parameters for the y-tube olfactometer bioassay that would later be used in spider olfaction trials. The fruit fly was chosen due to its similar mobility and size (1.59 mm) as the crab spider and the availability of various known attractants including our choices ethanol and ethyl acetate. [19-20]

### 2.4.1 Experimental

A y-tube olfactometer (Fig. 2.4) and sample delivery method was developed following Yan and Wang (2006). [21] Each choice arm of the y-tube measured 63.5 mm, and the entrance arm measured 0.127 m. Each choice arm was connected to a 50 mL Erlenmeyer flask with a two-holed rubber cork placed in each flask. One hole of the cork was connected to the y-tube using 0.44 m of Tygon tubing, and the other hole was attached to a small t-tube using 0.45 m of Tygon tubing. Both attractant and control flasks were attached to the same t-tube. A Tygon line of 0.90 m attached the base of the t-tube to a 500 mL Erlenmeyer flask containing 250 mL of water. Charcoal-purified air was passed into the water-containing flask at a rate of 21.81 mL/min. All Tygon tubing had a diameter of 6.35 mm. All flasks were secured with a ring stand.

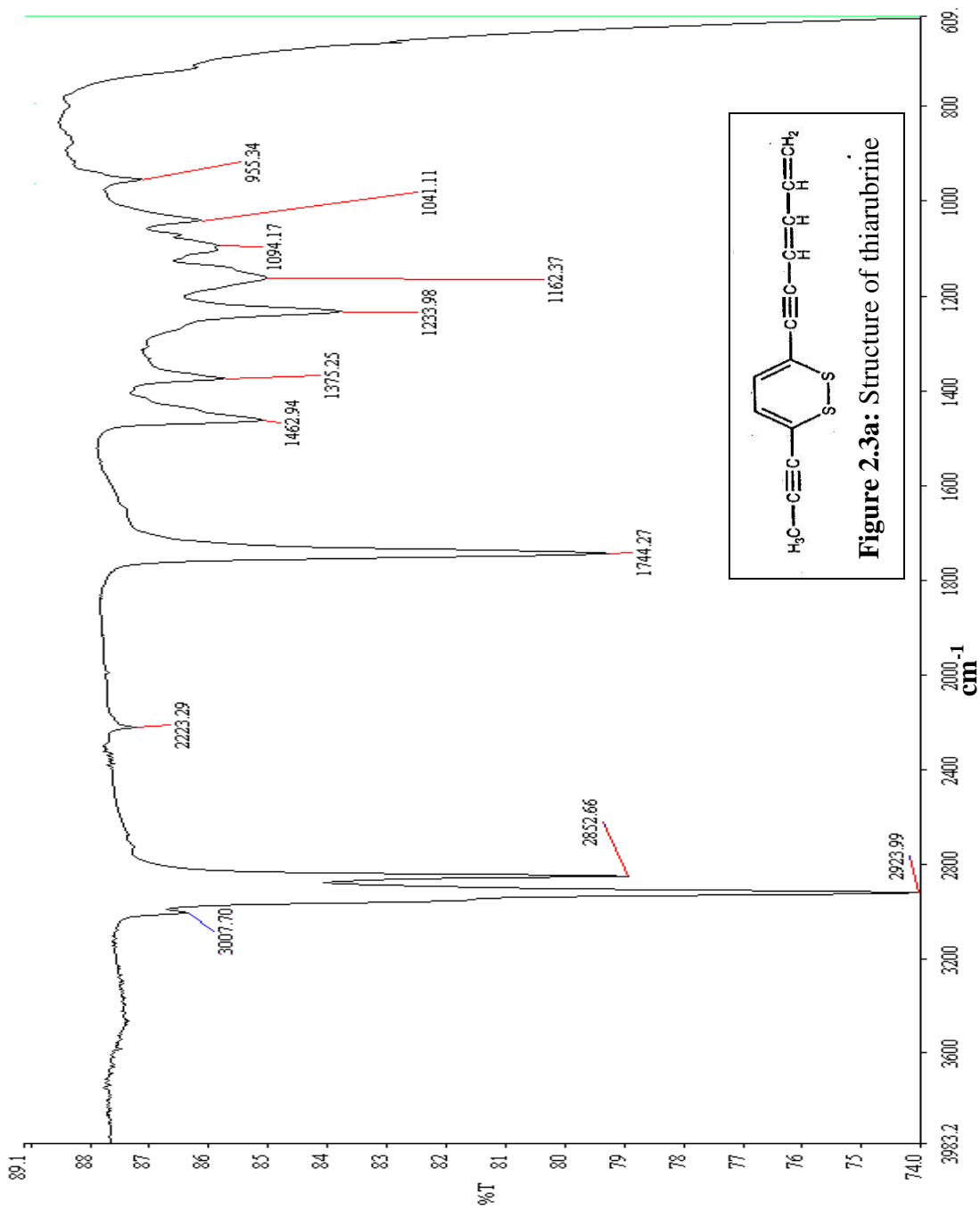


**Figure 2.1:** Infrared spectrum of 95% hexane pooled fractions 15-22



**Figure 2.2:** Infrared spectrum of 100% dichloromethane fraction 45





**Figure 2.3:** Infrared spectrum of 100% dichloromethane pooled 51-57 fractions

The attractant and control samples were delivered via evaporation from Whatman #2 filter paper cut to fit flat along the bottom of the flask. [21] Ten drops of sample were placed onto the filter paper, and the cork was placed into the flask. The tubing from the sample flasks was then attached to an arm of the y-tube. A white piece of paper was placed between flask and the y-tube in order to prevent any visual cues.

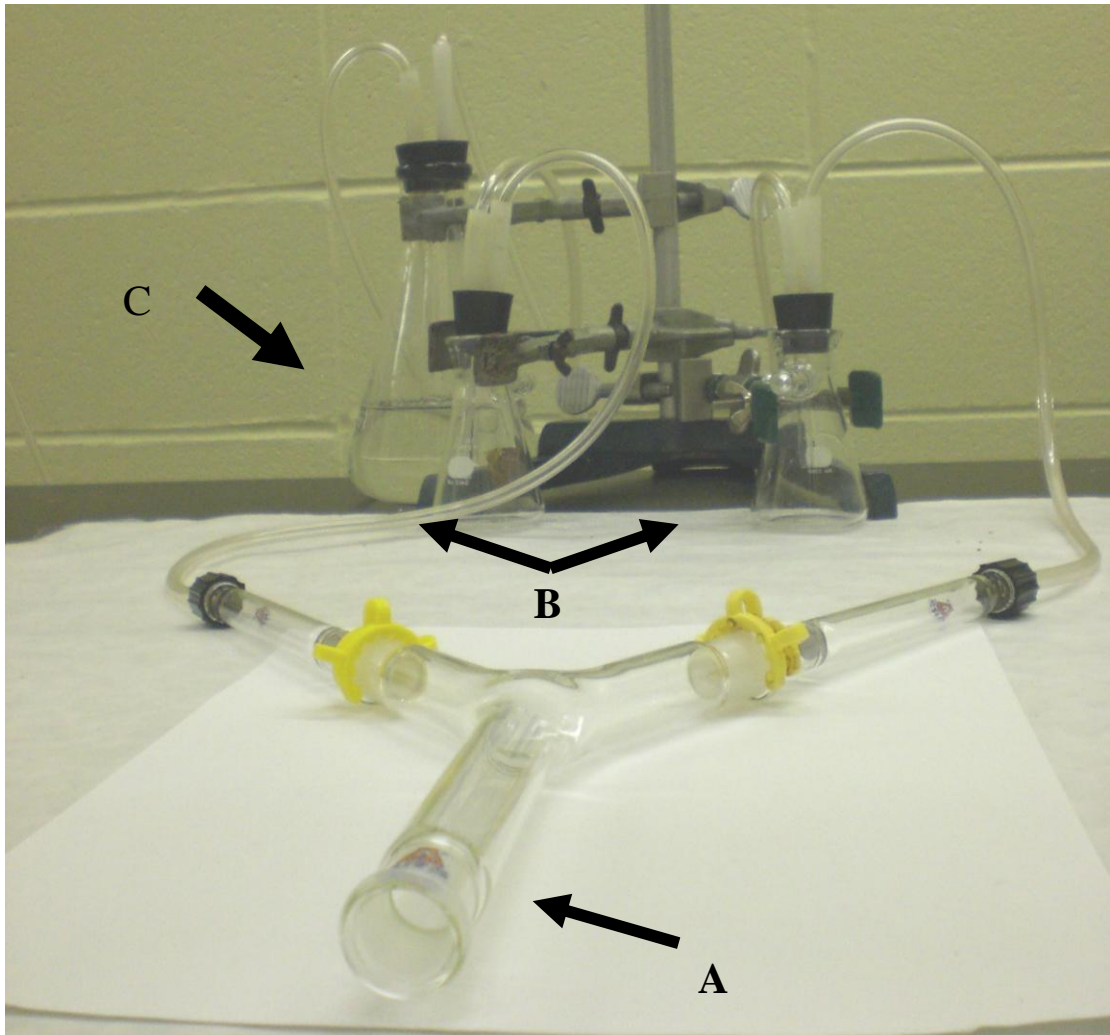
Each trial lasted 5 min and each fruit fly's first choice (attractant or control) was documented. A selection was determined once the fly crossed the interface of the air stream and entered a choice arm of the y-tube. The y-tube apparatus was washed with a soapy solution, rinsed and oven dried between each trial. The y-tube was flipped horizontally 180° and the attractant and control flask were switched from left to right arm of the y-tube.

Three fruit flies were placed together into the y-tube for each trial and 20 drops of attractant/control were applied to the filter paper. Fifteen fruit flies were tested with 95% ethanol as the attractant and water as the control. Twenty-six fruit flies were tested with a diluted ethanol solution (3:2 water:ethanol). Ninety-four fruit flies were tested with diluted ethyl acetate (3:2 water:ethyl acetate) as an attractant.

#### **2.4.2 Results and Discussion**

When 95% ethanol was used as an attractant the flies seemed to become immobile after traveling into the attractant arm. When the sample application was reduced to 10 drops of diluted ethanol 12 of 15 flies chose the ethanol arm.

Based upon the results of the diluted ethanol trials, additional studies were carried out with diluted ethyl acetate, and 66 of 104 flies chose the diluted ethyl acetate arm. The results of both the ethanol and the ethyl acetate trials indicated that the y-tube olfactometer could adequately assay olfactory behavior and would be used in crab spider olfaction trials. Purified-humidified air would be passed over the sample and control with an air flow rate of 21.82 mL/min, and ten drops of sample/control would be delivered to the spider via filter paper.



**Figure 2.4:** Y-tube olfactometer bioassay

A= Y-tube

B= Control and Attractant Flasks

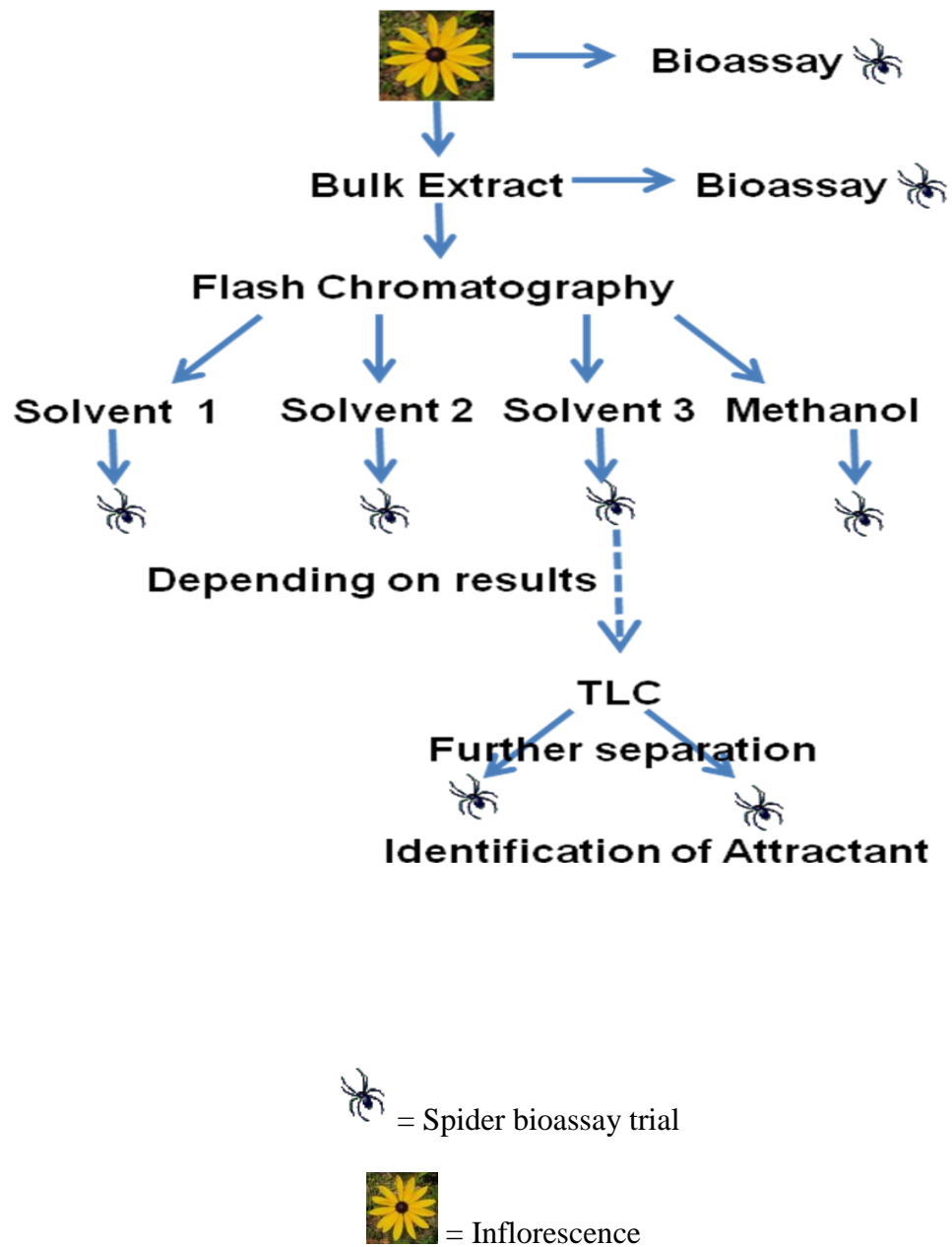
C= Humidifier

## Chapter 3

### *M. formosipes* Olfactory Response to *R. hirta*

Our experimental approach to determine if *M. formosipes* has an olfactory response to *R. hirta* inflorescences or their extract is shown in Figure 3.1. Positive bioassay trials using *R. hirta* inflorescences in the “attractant” flask would lead to bioassay trials performed on the bulk collection of components extracted from the inflorescence. *M. formosipes* preference for the bulk extract over the control would result in the extract being subjected to flash chromatography to separate some of the components using a series of solvents. Pooled fractions collected using each solvent system would then be tested in the bioassay.

Ultimately, if *M. formosipes* males are attracted to one or more of the pooled fractions, then further separation of those fractions would be necessary. By this process, we hoped to isolate and identify one or more potential chemical attractants in the inflorescences of *R. hirta*.



**Figure 3.1:** Flowchart of experimental approach

### 3.1 Experimental

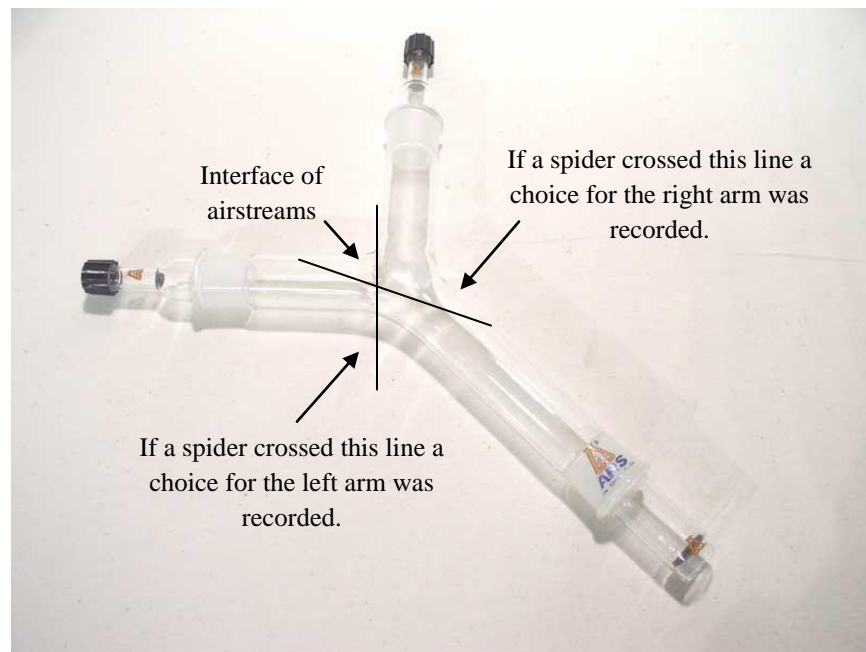
An ultrasonic-assisted extraction was performed on 10 g of *R. hirta* inflorescences, and the bulk extract was subjected to flash chromatography using the solvent system developed in Section 2.2.1, yielding a collection of thirty-two 9 mL fractions were collected. Based upon color of fraction, 7 pools were generated (Table 3.1). Those pools with color were tested and those without color were not tested.

<b>Pooled Fractions</b>	<b>Color of Pool</b>	<b>Tested in Bioassay</b>	<b>Mobile Phase</b>
<b>1-3</b>	Colorless	No	95% hexane
<b>4-5</b>	Pale Yellow	Yes	95% hexane
<b>6-12</b>	Colorless	No	95% hexane
<b>13-23</b>	Bright Yellow	Yes	95% dichloromethane
<b>24-26</b>	Colorless	No	100% dichloromethane
<b>27-32</b>	Yellow	Yes	100% dichloromethane
<b>33-35</b>	Yellow	Yes	Methanol

**Table 3.1:** Pooled Fractions

The y-tube olfactometer bioassay trials were conducted from August 5-19, 2008, and 75 spiders were tested. Male spiders and *R. hirta* inflorescences were collected daily

from Cooper Woods (Muncie, IN) and used within 24 h after collection. Each trial lasted for 10 h, and the duration of the trial was recorded using a video camera. If and when a spider crossed the interface of the air streams and entered one arm, a choice was recorded (Fig. 3.2). The y-tube was washed with a soapy solution, rinsed and oven dried between each trial.



**Figure 3.2:** Interface of airstreams from each arm of the y-tube. [12]

In *R. hirta* whole-inflorescence trials, 2 mL of water was placed in the attractant flask along with an inflorescence, and 2 mL of water alone was placed in the control



flask. In the remaining bioassay trials ten drops of the extract or pooled fractions were placed onto the filter paper in the “attractant” flask. In the bulk extract trials, 10 drops of the extraction solvent (1:2 hexane:diethyl ether) were put onto the control filter paper while in the pooled fraction trials the mobile phases used to collect each fraction pool were put onto the control filter paper.

### 3.2 Results and Discussion

Responses by the male spiders varied across attractant categories (Table 3.2). Exact binomial probabilities of obtaining the results for each set of trials in relation to chance were calculated using the equation:

$$P_{(k \text{ out of } n)} = \frac{n!}{k!(n-k)!} (p^k)(q^{n-k})$$

where k is the number of times the spider chose the “attractant” for a particular trial type, p is the probability that the spider will choose the “attractant” (0.5), q is the probability that the spider will choose the “control” (0.5), and n is the number of trials in which a spider made a choice. A low P value therefore is indicative of a low probability that the spiders made their choice due to random movement.

Statistically significant choices of the attractants were found for the inflorescences, bulk extract, and the 100% dichloromethane pool. Given this result, the 100% dichloromethane pool was subjected to further separation to isolate the potential chemical attractants.

Mulberry leaves were tested as a potential attractant using the same procedure as the inflorescence trials. The purpose was to test the hypothesis that *M. formosipes* males would be attracted to any plant organic matter in comparison with the water control. No attraction to the leaves was found, but more trials will have to be run to test the idea.

The number of trials completed is directly proportional to the statistical significance of the P value. It is possible that the P value calculated for each of the trials could increase or decrease depending upon the outcomes of future trials. For that reason, additional bioassay trials will be carried out on the pooled fractions.

<b>Type of Trial</b>	<b>Number of Spiders Tested in Bioassay</b>	<b>Number of Spiders Choosing Potential Attractant</b>	<b>Number of Spiders Choosing Control</b>	<b>Number of Spiders Not Choosing an Arm</b>	<b>P value</b>
<b>Inflorescences</b>	21	16	3	2	.0018
<b>Bulk Extract</b>	18	12	5	1	.0472
<b>95% Hexane Pool</b>	6	4	2	0	.2344
<b>95% Dichloromethane Pool</b>	6	3	3	0	.3125
<b>100% Dichloromethane Pool</b>	9	8	1	0	.0176
<b>Methanol Pool</b>	6	1	3	2	.2500
<b>Mulberry Leaves</b>	6	2	4	0	.2344

**Table 3.2:** Bioassay trials results

## Chapter 4

### **Separation and Identification of the Possible Attractants in the 100% Dichloromethane Fractions**

The y-tube olfactometer bioassay trials provided strong evidence that the pooled 100% dichloromethane fractions contain components that are attractive to male *M. formosipes*. Consequently, the 100% dichloromethane pool was the focus of the remainder of the research. This pool was subjected to flash chromatography to facilitate further separation of components. Fractions that showed evidence of containing components were subjected to FT-IR and NMR spectroscopy in order to determine the types of compounds present.

#### **4.1 Separation of 100% Dichloromethane Pooled Fractions from Bioassay Trials**

##### **4.1.1 Experimental**

As described in Section 2.1.1 flash chromatography was performed on the pooled 100% dichloromethane fractions that showed attraction in the bioassay trials. A total of eighteen 5 mL fractions were collected. Fractions 1-5 were collected with 95% hexane, 6-10 were collected with 95% dichloromethane, 11-15 were collected with

100% dichloromethane and 16-18 were collected with methanol. TLC was carried out on each of the fractions as stated in Section 2.1.1

#### 4.1.2. Results and Discussion

Those fractions with similar  $R_f$  values were pooled together; a total of 2 pools were generated (Table 4.1).

<b>Pooled Fractions</b>	<b><math>R_f</math> value</b>	<b>Mobile Phase</b>
<b>11-12</b>	1	100% dichloromethane
<b>13-15</b>	.8	100% dichloromethane

**Table 4.1:** Pooled 100% dichloromethane fractions

All of the fractions collected were colorless, except for those collected with methanol. The 100% dichloromethane fractions were the only fractions that showed components upon staining. To determine the types of compounds present FT-IR and NMR were performed on the pooled fractions.

## 4.2 Spectroscopy on 100% Dichloromethane Pooled Fractions from Bioassay Trials

### 4.2.1 Experimental

FT-IR was performed as described in Section 2.3.1 on the two pools of fractions, as well as on the entire collection of 100% dichloromethane fractions (11-15). All IR spectra obtained were manually baseline corrected. Proton NMR spectroscopy was carried out on the same sample. The solvent used was  $d_2$ - $CH_2Cl_2$ , and 10 co-added scans were taken on a 400 MHz NMR spectrometer.

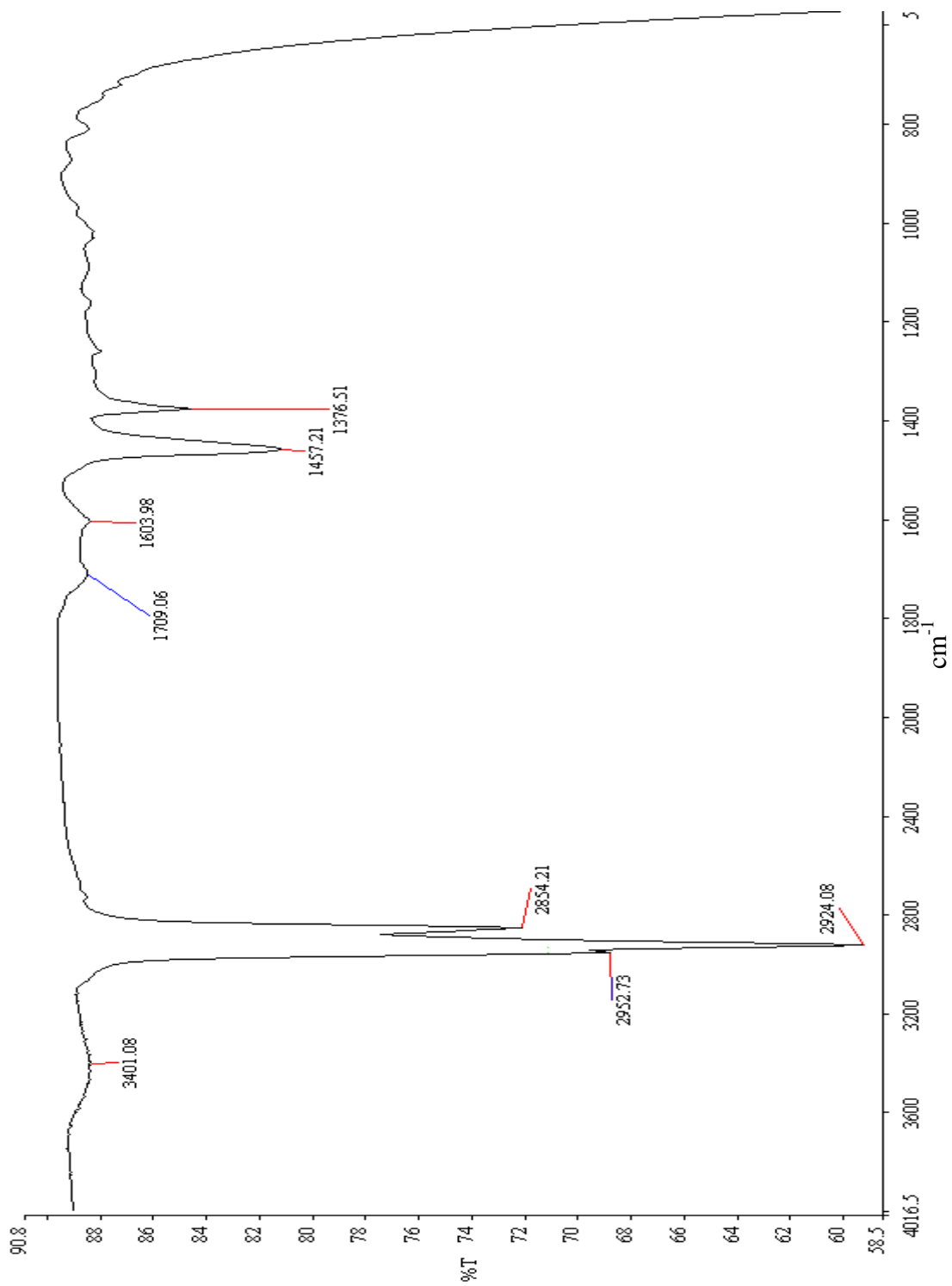
### 4.2.2. Results and Discussion

Figure 4.1 is an IR spectrum of the entire collection of 100% dichloromethane fractions (11-15). The spectrum contains methyl and methylene stretching ( $2854$ - $2953$   $cm^{-1}$ ) and bending modes ( $1377$   $cm^{-1}$ ,  $1457$   $cm^{-1}$ ). It also has an OH stretch at  $3401$   $cm^{-1}$ . There is a band at  $1604$   $cm^{-1}$  which is consistent with benzene ring stretching. In addition, this spectrum contains a broad absorption at  $1709$   $cm^{-1}$  which is consistent with a C=O stretch. Due to the relatively low frequency we propose that it could correspond to a ketone. Since this band is also broad it could possibly indicate that a higher frequency C=O absorption, such as an ester, is also present. The relative intensities of the C=O absorption compared to the methyl and methylene stretching suggest a very long hydrocarbon chain compound. Figure 4.2 is the proton NMR spectrum of the same sample. The solvent peak from dichloromethane appears at 5.3 ppm, and water (HOD) in the solvent has a peak at 1.51 ppm. Methyl and methylene peaks show at 1.25 ppm and .865 ppm respectively. The very weak, broad, and low intensity peaks showing around 7 ppm are consistent with aromatic protons. The 2-2.5 ppm peaks near the baseline are

consistent with methylene and/or methyl groups that are substituents on a benzene ring. The relative intensities of benzene group absorption to the methyl and methylene absorption observed in both spectra suggest that a benzene compound is a minor component.

The IR spectrum of pooled 100% dichloromethane fractions 11-12 is shown in Figure 4.3, and Figure 4.4 is the IR spectrum of pooled 100% dichloromethane fractions 13-15. Each of the spectra shows evidence of methyl and methylene stretching and bending modes. However, Figure 4.3 appears to be primarily a long chain hydrocarbon (comparable to heptadecane), whilst Figure 4.4 has an additional absorption at  $1705\text{ cm}^{-1}$  which is consistent with a ketone C=O stretch. Figure 4.4 also has an absorption at  $1605\text{ cm}^{-1}$  that could possibly indicate a ring stretch, consistent with a benzenoid compound present in a small amount. We also observe an absorption at  $1773\text{ cm}^{-1}$  which is consistent with a lactone C=O stretch. The broadness of the C=O absorption could indicate a C=O stretch of an ester as well.

These spectra suggest that we were successful in separating a long chain hydrocarbon from other types of compounds. This data was consistent with NMR data on the pooled fractions. As a result, a separation was performed on another *R. hirta* extract to see if better separation can be achieved.



**Figure 4.1:** Infrared spectrum of 100% dichloromethane fractions 11-15



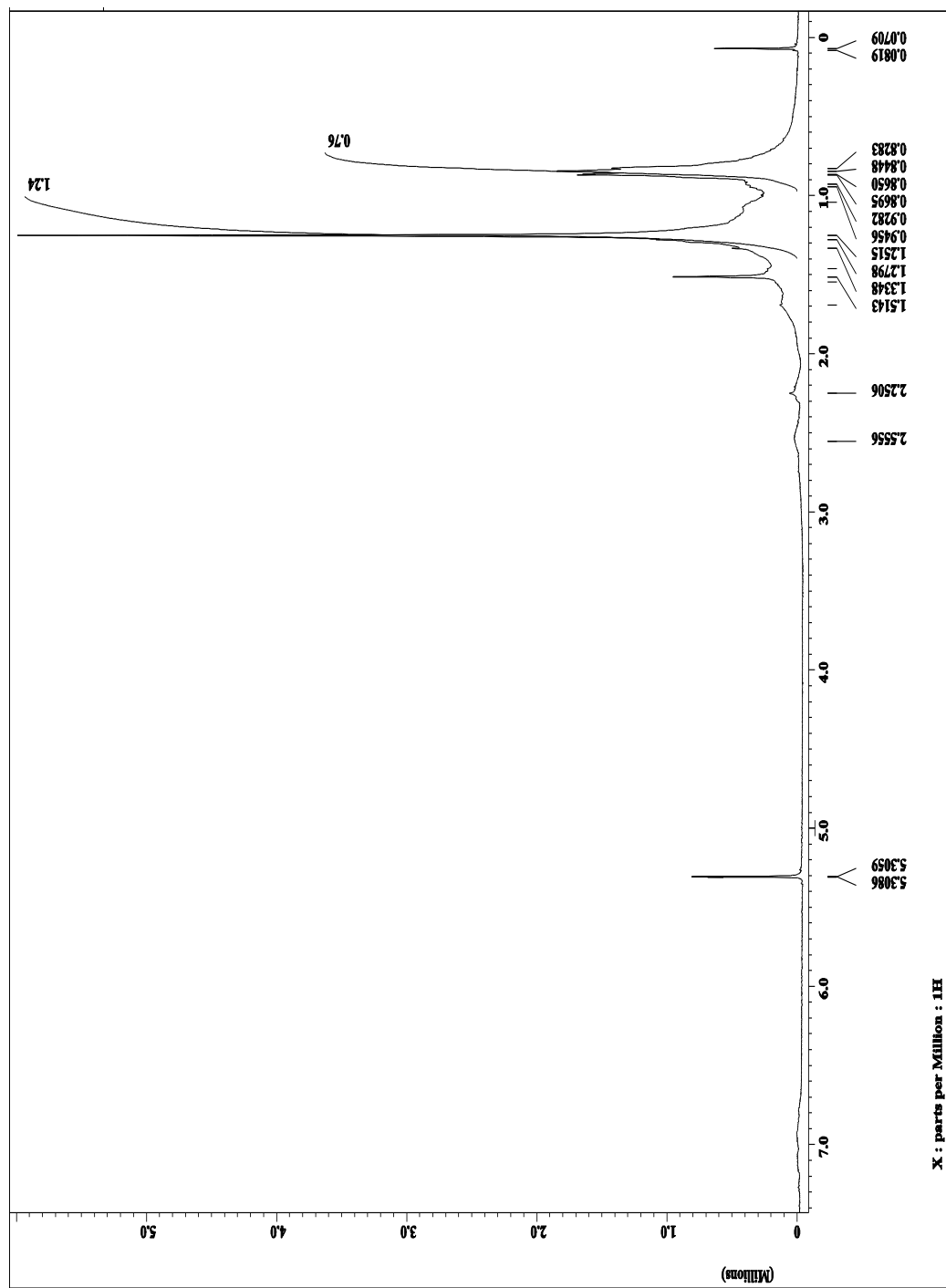
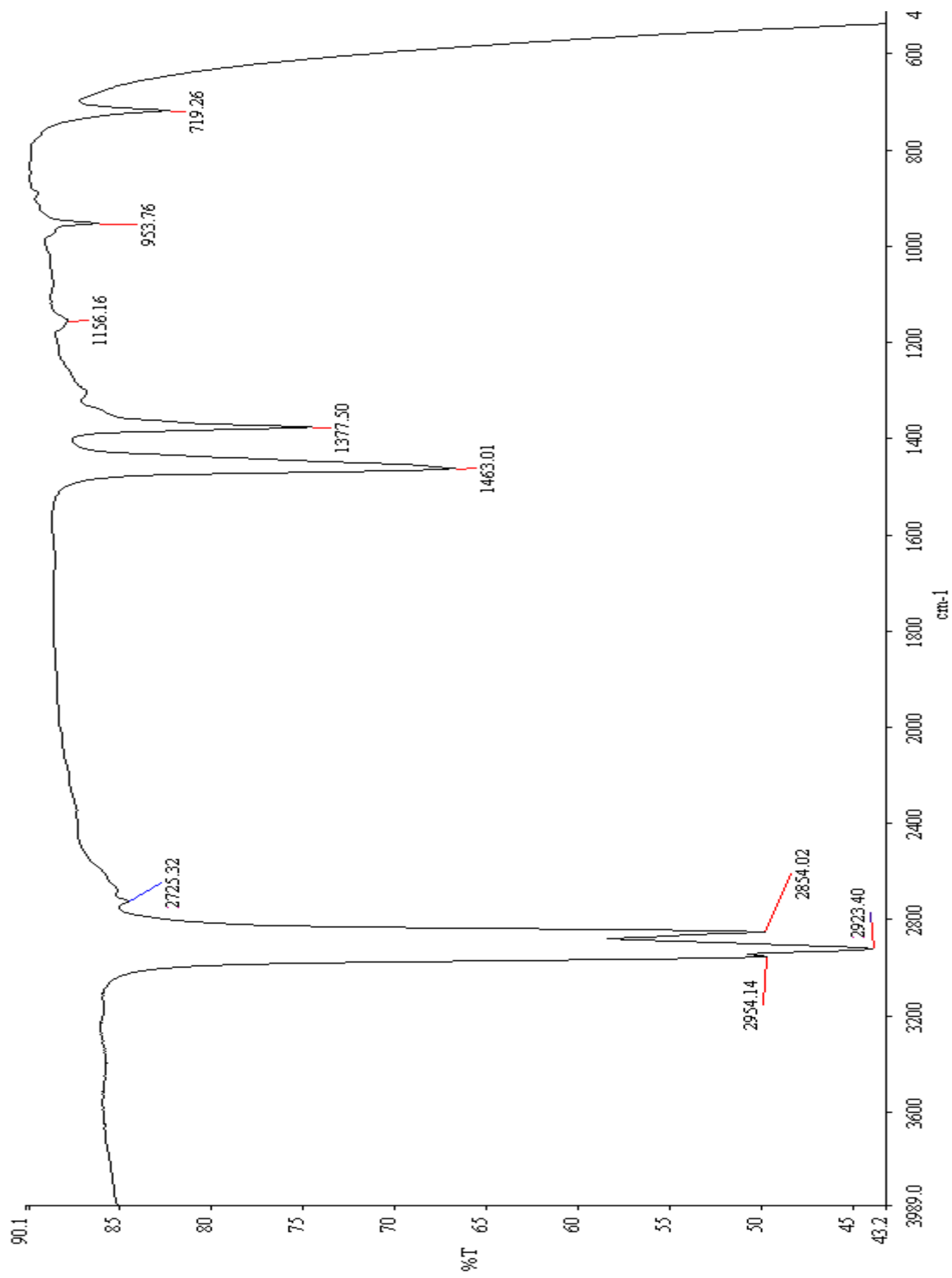
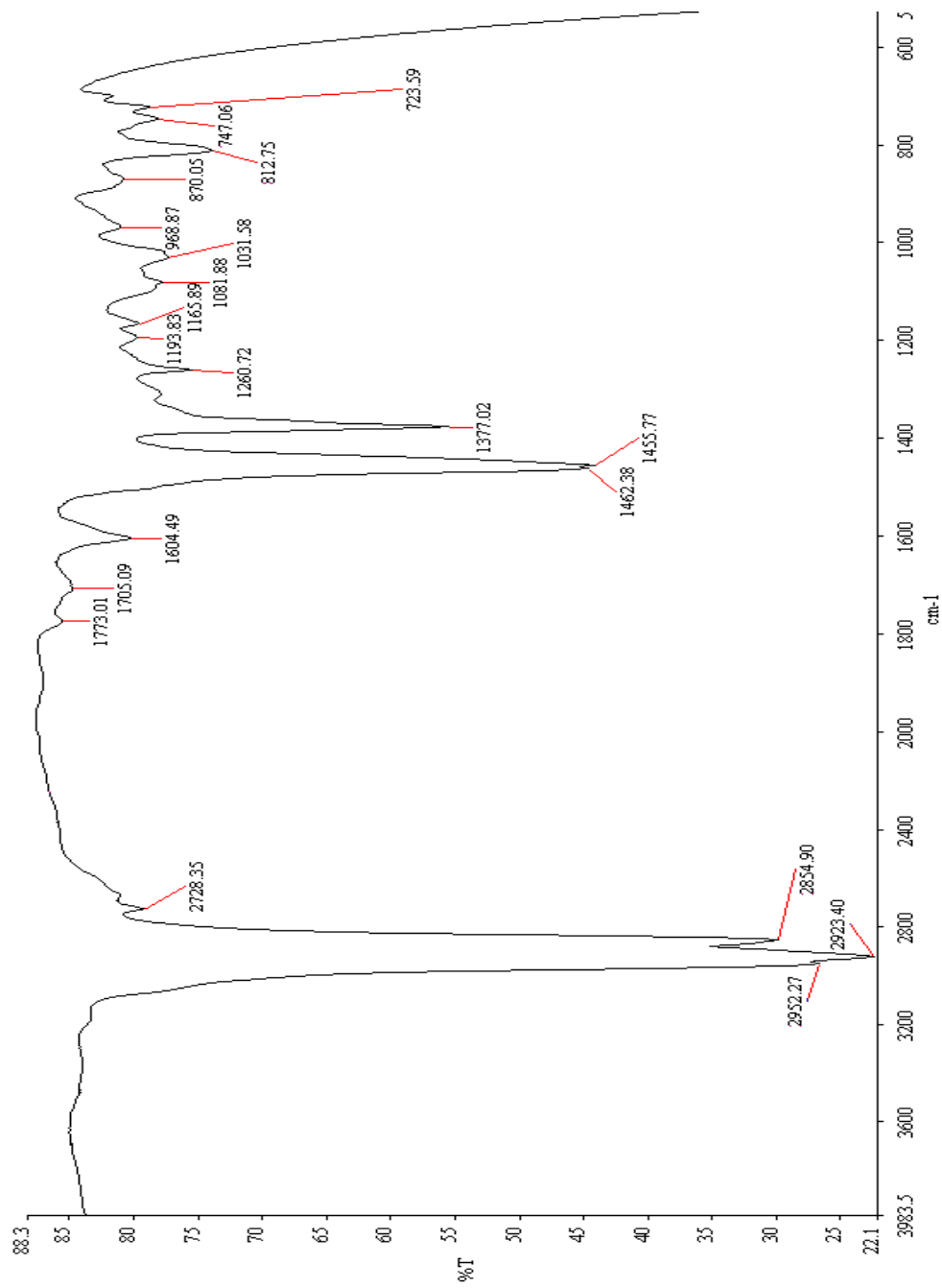


Figure 4.2: Proton NMR of pooled 100% dichloromethane fractions 11-15



**Figure 4.3:** Infrared spectrum of 100% dichloromethane pooled fractions 11-12



**Figure 4.4:** Infrared spectrum of 100% dichloromethane pooled fractions 13-15

### 4.3 Analysis of 100% Dichloromethane Fractions from Frozen *R. hirta* Extract

#### 4.3.1 Experimental

An ultrasonic extraction was performed on *R. hirta* inflorescence as described in Section 2.2.1. This extract was kept frozen (approximately 8 months) until it was subjected to flash column chromatography using the procedure stated in Section 2.2.1. A total of sixty-six 9 mL fractions were collected. Twenty fractions were collected of each solvent (95% hexane, 95% dichloromethane, and 100% dichloromethane) and 6 were collected using methanol. TLC was performed on the 100% dichloromethane fractions as stated in Section 2.1.1. These pools were subjected to FT-IR and proton NMR spectroscopy as stated in Section 2.3.1 and Section 4.2.1, except  $d\text{-CHCl}_3$  was used as the solvent instead of  $d\text{-CH}_2\text{Cl}_2$ .

#### 4.3.2 Results and Discussion

The 100% dichloromethane fractions were pooled together based upon similar  $R_f$  values. A total of five pools were generated (Table 4.2). Characteristic IR bands of each pool are listed in Table 4.3 and all NMR peaks of each pool are listed in Table 4.4. Figure 4.5 is the proton NMR spectrum of the pooled fractions 44-48. Unlike the NMR spectrum shown in Figure 4.2 the spectrum suggest that a long chain fatty acid ester comparable to ascorbic acid- dihexadecanoate (see [http://riodb01.ibase.aist.go.jp/sdbs/cgi-bin/direct\\_frame\\_top.cgi](http://riodb01.ibase.aist.go.jp/sdbs/cgi-bin/direct_frame_top.cgi)) is the major component in the fraction pool, and the minor component could possibly be a lactone. In addition, an alcohol may also be a minor component. Table 4.3 lists the significant IR bands and Table 4.4 lists methylene and

methyl NMR peaks of each of the pooled 100% dichloromethane fractions.

<b>Pooled Fractions</b>	<b>R<sub>f</sub> value of each fraction</b>
<b>41-43</b>	.50
<b>44-48</b>	.40
<b>49-51</b>	.32
<b>52-53</b>	.28
<b>54-60</b>	.30

**Table 4.2:** Pooled 100% dichloromethane fractions based on similar R<sub>f</sub> values.

Pooled Fractions	Infrared Assignments cm <sup>-1</sup>							
	v(OH)	v(CH <sub>3</sub> /CH <sub>2</sub> ) stretching	v(C≡C) or v(C≡N)	v(C=O) lactone	v(C=O) ester	v(C=O) ketone	v(C=C) v(C≡O)	v(CH <sub>3</sub> /CH <sub>2</sub> ) bending
41-43	3350	2956-2849	NP	1770(sh)	1732(br)	NP	NP	1463, 1379
44-48	3401	2965-2849	2253	1774	1728	NP	1642(br)	1462, 1378
49-51	3436	2957-2849	NP	1780	1732	NP	1638(br)	1463, 1378
52-53	3585* 3539	2956-2851	NP	NP	1732(br)	NP	NP	1463, 1377
54-60	3537	2957-2851	NP	NP	1733	1717(sh)	NP	1463, 1377

\*Non-hydrogen bonded  
NP= not present

**Table 4.3:** IR spectra bands of pooled 100% dichloromethane fractions

Pooled Fractions	NMR Assignments ppm		
	Methylene Peaks	Methyl Peaks	Approx. Peak Intensity Ratio (CH <sub>2</sub> /CH <sub>3</sub> )
41-43	1.22(s), 1.24(s), 1.33(s)	.860(br) Fine structure not present	3:1
44-48	1.24(s), (1.27(s), 1.32(s))*	.865(multiplet)	4:1
49-51	1.25(s), (1.27(s), 1.32(s)) very weak	.878(br) Fine structure not present	4:1
52-53	1.24(s), (1.27(s), 1.32(s)) very weak	.837(br) Fine structure not present	3:1
54-60	1.256(s), (1.29(s), 1.34(s))	.845(br) Fine structure not present	3:1

\*much weaker than previous pooled fractions (41-43)

Table 4.4: NMR spectra peaks of pooled 100% dichloromethane fractions

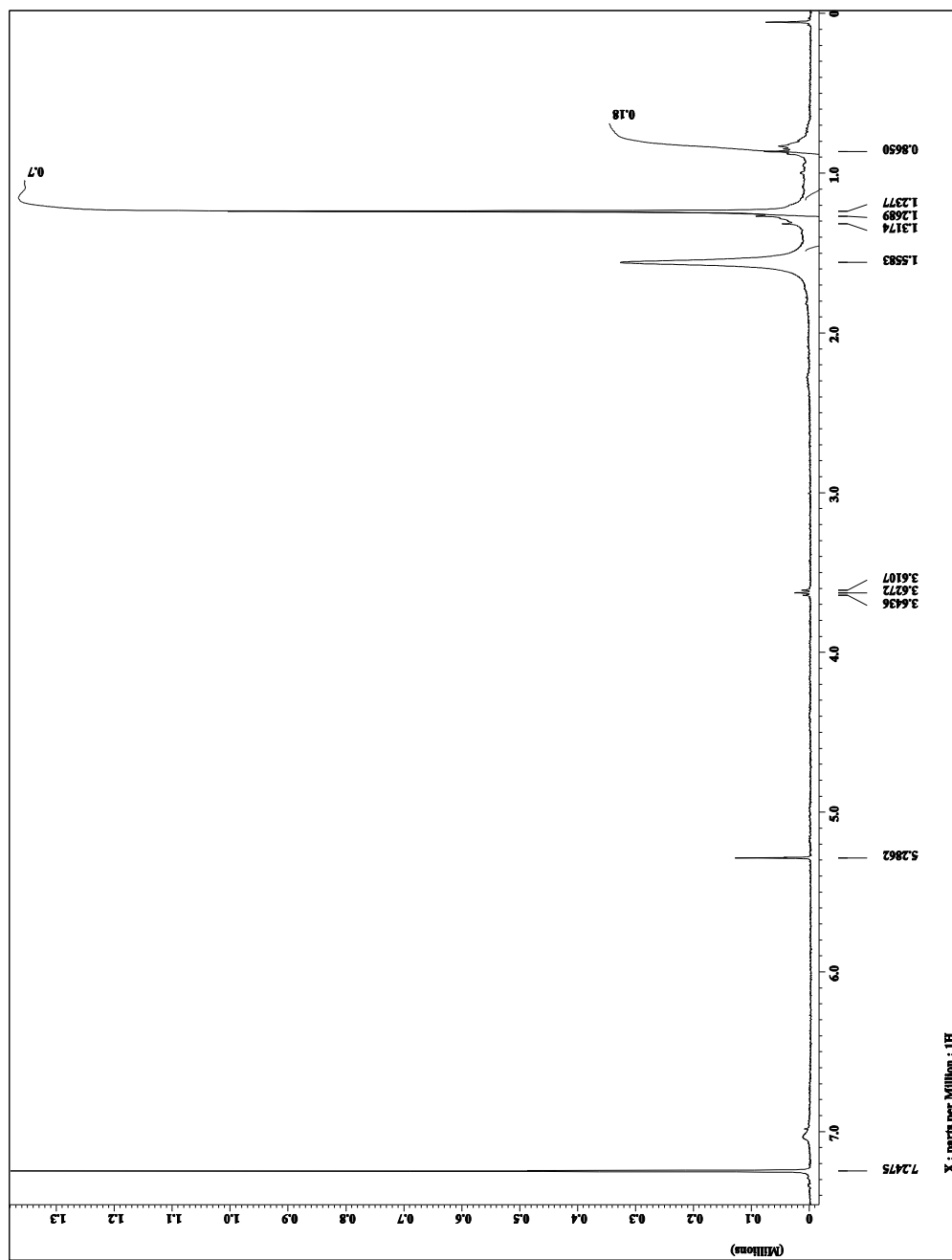


Figure 4.5: Proton NMR of pooled 100% dichloromethane fractions 44-48



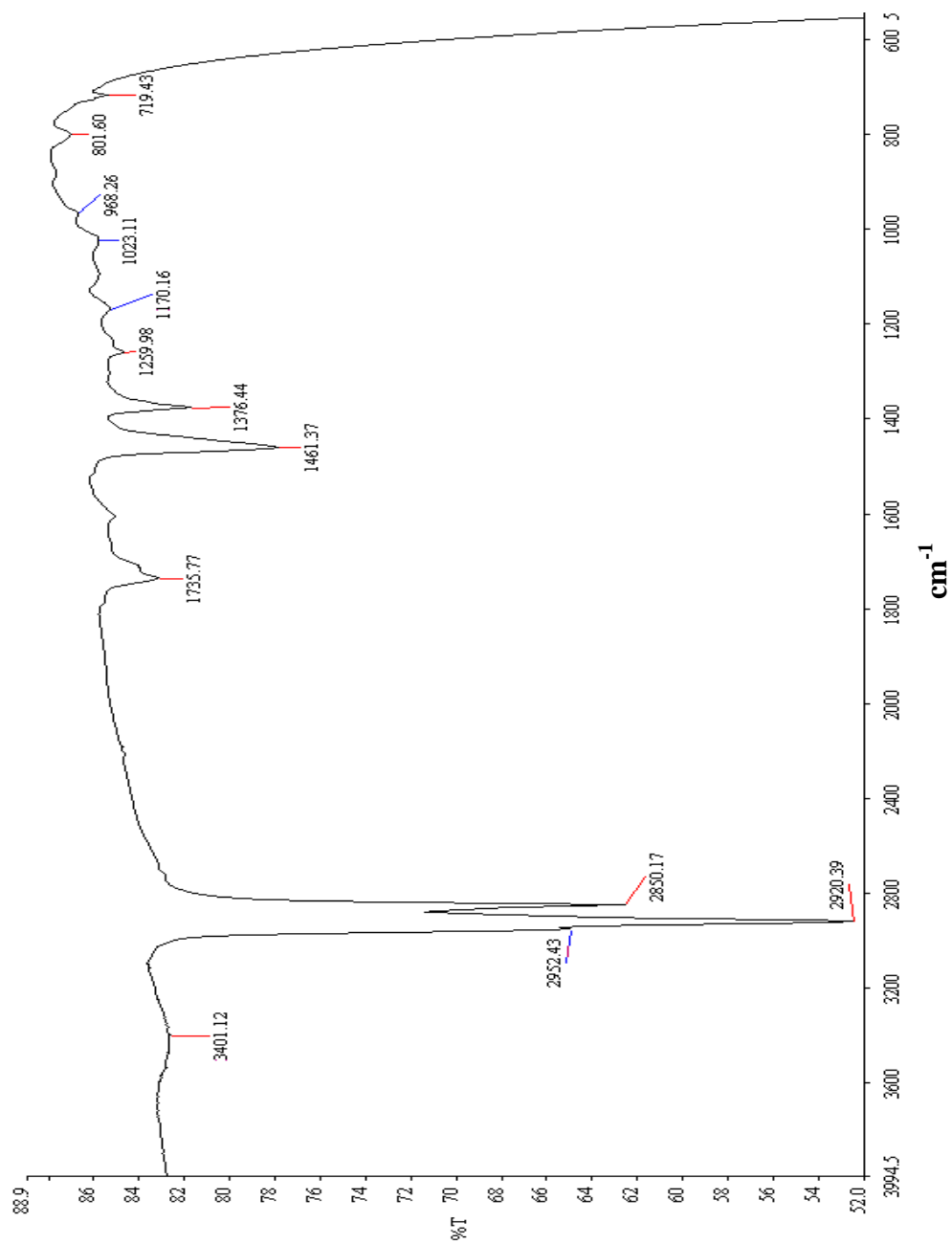
#### 4.4 Conclusions

Y-tube olfactometer bioassay trials provided strong evidence that male *M. formosipes* crab spiders are attracted to *R. hirta* inflorescences through olfactory cues. These trials also suggested that components obtained from flash column chromatography with 100% dichloromethane as the mobile phase are attractive to the spider. After performing FT-IR and NMR spectroscopy experiments on the 100% dichloromethane pooled fractions, it is evident that the ultrasonic- assisted extraction was successful in extracting components from the inflorescences. Spectroscopic analysis also provided strong evidence that a long chain hydrocarbon is the main component in the 100% dichloromethane pool. This finding is consistent with reports of long chain hydrocarbon volatiles extracted from *Echinacea* species. [22] In all inflorescences (frozen and fresh), the long chain hydrocarbon was present. These trials also suggest that the minor components present vary amongst inflorescences. In addition to these studies, comparison of infrared spectra taken of two inflorescence bulk extracts reveal different amounts of esters and ketones in *R. hirta* inflorescences (Fig. 4.6 and 4.7). These data suggest that plants of the same species can have inconsistencies in the quantity and types of minor volatile compounds emitted. These differences could possibly be attributed to plant defense mechanisms, atmospheric CO<sub>2</sub> levels, soil and air humidity, temperature, light intensity, age of plant, and plant-plant interactions. [23]

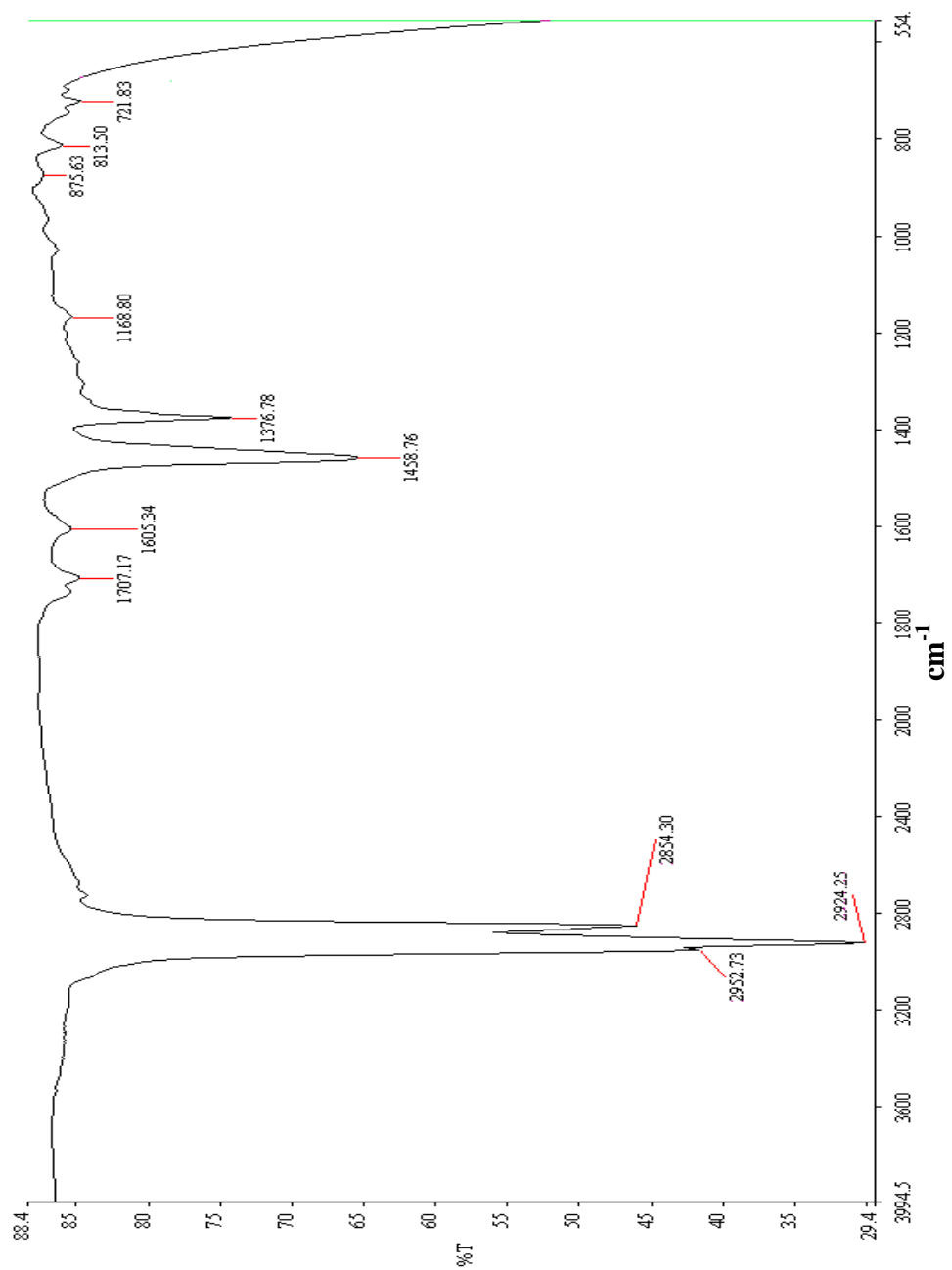
Due to limited number of trials completed, for some categories additional bioassay trials will help clarify the exact nature of the volatile attractant(s). Future trials will aide in determining if the 100% dichloromethane fraction pool contains an attractant.

In addition, they will help to determine if other pools of fractions that were collected with the other solvents contain attractants.

Once a pool is determined to be an attractant, other analytical techniques such as, GC-MS, along with FT-IR and NMR spectroscopy can be performed to aide in identifying the chemical attractants. Unfortunately, GC is not a trivial task. GC-FID was attempted in order to further determine the number of components present in the 100% dichloromethane pool; however, reproducible results could not be obtained after several attempts. Therefore, further method development is necessary.



**Figure 4.6:** Infrared spectrum of bulk *R. hirta* extract collected on 8/10/08



**Figure 4.7:** Infrared spectrum of bulk *R. hirta* extract collected on 8/20/08

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