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ANNUAL REPORTRaman Spectroscopy to Detect and Measure NOW Pheromones

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ANNUAL REPORT
Raman Spectroscopy to Detect and Measure NOW Pheromones

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Introduction

Insect sex pheromones are chemical compounds that insects release to attract mates over distances of hundreds of meters or even kilometers, in complete darkness and without any audible signals. Use of synthetic forms of key compounds have in some cases become an essential component of monitoring and/or managing key pests of agricultural crops, including navel orangeworm (Amyelois transitella) (NOW) in California tree nuts [1-3] There are currently multiple commercially available mating disruption products available for NOW and recent studies have demonstrated that they can be effectively used to reduce crop damage [4]. How mating disruption works is not fully established and likely varies across products and target species [5]. For instance, it is not well understood how synthetic pheromones compete with natural pheromones and, in the case of monitoring, how efficaciously the insect follows the diffusing plumes and how those evolve from emission points, especially across large blocks and at plot borders. We proposed to evaluate the use of enhanced Raman spectroscopy [6,7] for the detection of synthetic and natural NOW pheromones and, were that to be successful, use it in subsequent years to measure pheromone diffusion in orchards.

We have continued from Year 1 on targeting two main objectives:

Objective 1–Use Raman to Detect Pheromones.

Objective 2–Use Raman to Measure Pheromone Diffusion.

In Year 1 we were able to generate the Raman signatures for the main chemicals [8, 9] in synthetic pheromones, and now in Year 2 we have been able to provide a clear distinction of such signatures in mixed samples via Principal Component Analysis (PCA) [10], strengthening our conclusions on the ability of Raman spectroscopy to detect emissions in more realistic conditions. In Year 2, we also continued our preparative efforts in measuring any release from dispensers or lures and have setup some of our first "orchard in a box" experiment for diffusion studies. Given the low concentrations of pheromones released, we pursued boosting the detection capacity by implementing two approaches: (1) preconcentrate with Solid Phase Micro Extraction (SPME) fibers following GC-MS approaches [11] applied to Raman; and (2) pursue Surface Enhanced Raman Spectroscopy (SERS) to enhance the overall optical signals [7]. Diffusion experiments were started too.

Year 2 funding from CPRB was only approved within LLNL in May 2021, causing delays, that were further accentuated by lingering COVID-19 crisis, since laboratories had limited access until July 2021. During this downtime we requested and received DoE and State approval to work with live NOW moths for future experiments in collaboration with Dr. Wilson at UC Riverside. Despite all these constraints, we generated new promising data on Raman selectivity and on pathways for enhancing its sensitivity, to provide a tool for concentration-dependent diffusion studies of synthetic vs natural pheromones.

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Objective 1 – Use Raman to Detect Pheromones

Since in Year 1 we had demonstrated the ability to detect Raman signals for various concentrations of what are accepted as most critical components in pheromone, i.e. ((Z,Z)-11,13-Hexadecadienal, (Z,Z,Z,Z,Z)-(3,6,9,12,15)-Tricosapentene, and (E,Z)-7,9-Deocedadien-1-yl Acetate1,2 [8,9], this year we extended our Raman exploration to mixtures diluted in hexane or acetone. Our Raman system works in reflection, and comprises a 785nm, 30mW power source with a built-in spectrometer with ~10cm-1 resolution. The portable gun aims at the core of a built-in vial holder where the vials with the chemicals, or lures/dispensers/moths, can be set. This configuration offers the best focusing of the laser beam on the samples and thus best Signal-to-Noise ratio. We pursued Raman concentration calibration of the basic chemical components down to 0.1% in acetone and hexane, enabling the possibility to evaluate their mixtures, like shown in Figure 1A for mix #1 and Figure 1B for mix #2. The mixtures clearly show variations in the spectrum from a single component. The complex signatures were also analyzed with Principal Component Analysis (PCA) [10] to help isolating the response of each mixture and differentiating the signatures (Figure 1C). PCA is a multivariate method of analysis that helps reducing the dimensionality of a multi–dimensional data set comprising many variables, while retaining those characteristics of the original data set that contribute most to its variance. PCA is an orthogonal linear transformation of the original data into a new basis with new coordinates (Principal Components – PCs) that are mutually orthogonal and ordered such that the first few variables exhibit the greatest amount of variation, and the remaining may be disregarded (thus allowing for a massive reduction of data – with no relevant loss in information). In Figure 1C we have outlined 3 boxes, the first two (1 and 2) identify the single components and the box 3 represents the mixtures of the different chemicals diluted in acetone. In the plot, each mixed sample falls in the areas between the single chemicals, closer to one or another depending on the mixed levels. By varying concentrations of the chemicals in mix #1, we were able to easily discriminate the various samples, validating the power of the PCA vs simple Raman peak analysis. We are extending the study to trend simple mixtures concentrations and to larger than 3 components mix to be more representative of the natural (or synthetic) pheromone emitted profiles. In Figure 3D we provide our preliminary results for mixtures trends for mix#1 (large green dashed circle), and mix#2 (large brown dashed circle) at various concentrations, overlayed with full mix of the 3 chemicals at 12.5% each in acetone (yellow circle) which falls at intersection of the main components. More data processing is required for helping to highlight the trends, but the different mixtures can be identified by location in the P1-P2 score plots. These data can be used for trending and calibration for determining unknown concentrations in the fields. A database of such mixtures will be built for reference.

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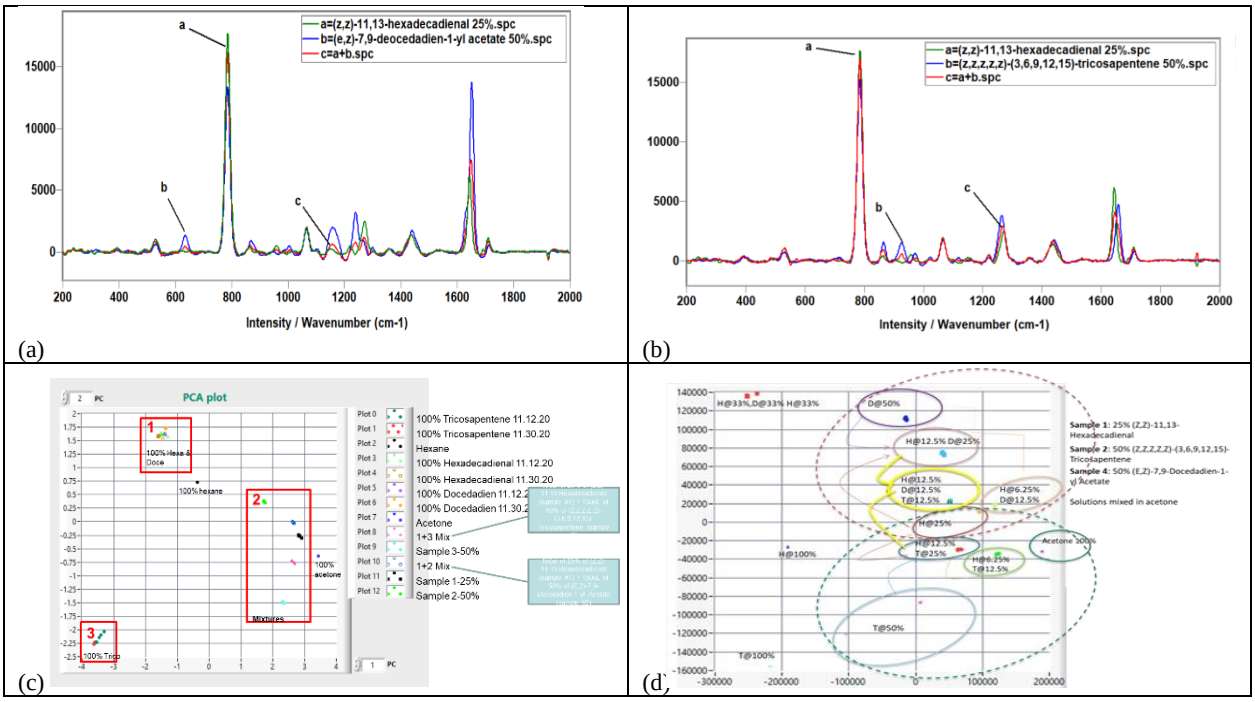
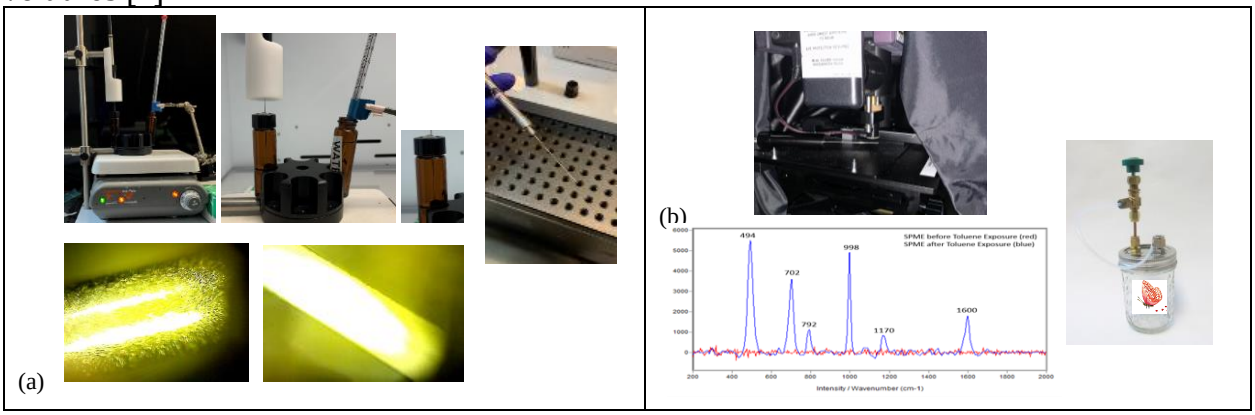


Figure 1. Spectra of mixed samples: **(*A*) mix #1**: 150uL of 25% of (Z,Z)-11,13-Hexadecadienal and 150uL of 50% of (E,Z)-7,9-Deocedadien-1-yl Acetate; **(*B*) mix #2:** 150uL of 25% of (Z,Z)-11,13-Hexadecadienal and 150uL of 50% of (Z,Z,Z,Z,Z)-(3,6,9,12,15)-Tricosapentene; ***(C)*** PC1 vs PC2 represent clear distinction between the single components and the mixtures. ***(D)*** mixtures trends for samples 1, 2, and 3 at various concentration and their mix at 12.5% (YELLOW CIRCLE) which falls at intersection of main components.

Having confirmed the validity of Raman detection of the chemicals as provided by the vendors, the next effort has been to evaluate them in gas phase. Our preliminary tests with various lures (Pherocon L2L, L2H by Trece) contained in vials were not conclusive likely because of extremely low concentration they released and leakages through the vials. We then moved on to mating disruption dispensers since they have 10-100x higher emission rates [CIDETRACK, Trece] and we just started testing them. We are also tackling this challenge via increasing the sensitivity via preconcentration: (1) of chemicals via Solid Phase Micro Extraction (SPME) fibers, following the examples with GC-MS [11] and (2) of light via Surface Enhanced Raman Spectroscopy (SERS), following examples for other organic volatiles [7].



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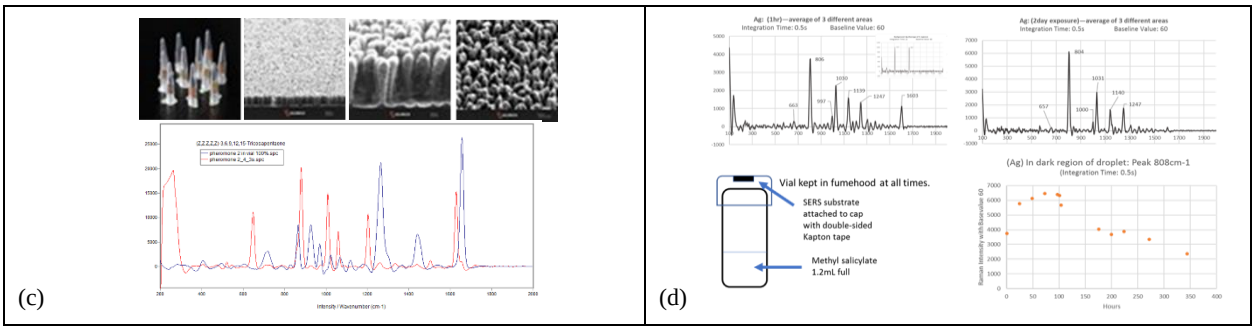

Figure 2. ***(A)*** SPME test setup: fibers exposed just to the headspace of the vial on hotplate, partially filled with toluene; ***(B)*** results of measured toluene onto SPME fiber itself (shown under Raman gun) and concept for SPEM with moths; ***(C)*** Raman of SPME before (blue plot) and after (red plot) exposure to toluene for 5 minutes at 130C indicating successful uptake; (c) (Z,Z,Z,Z,Z)-3,6,9,12,15-Tricosapentaene on Ag substrate; ***(D)*** example of previous work on smelling the nuts breath

1) SPME fibers appropriate for the molecular weights of semiochemicals (50/30um DVB/CAR/PDMS, Stableflex 24Ga by Sigma-Aldrich) were first tested with diluted toluene uptake, as a reference/baseline and revealed to be successful as shown in the plots of Figures 2A and 2B. Eventually, the SPME will be inserted in the headspace of a vial containing calling moths as shown in Figure 2B as well. The second part of the test, where we heat up the SPME fiber in another vial empty headspace to release the adsorbed toluene, did not provide any meaningful results yet because we did not reach the necessary temperature (>200C) to activate the process. We are procuring a new hotplate with better temperature controls. We're now verifying headspace release and semiochemical uptake at >200C

2) In parallel, we also commenced exploring SERS for increasing the sensitivity. The nanopatterned leaning pillars introduce many hotspots that augment light shining onto and scattering back from the molecules. After validation and calibration of SERS substrate with more standard molecules (bipyridylethylene, and toluene) we obtained preliminary results for semiochemicals as shown in Figure 2C. The SERS peaks are shifted from bulk Raman ones since the vibrational modes are affected by the affinity of the molecules to the substrate and how they orientate themselves on it. More tests and analysis are still necessary and ongoing to confirm trends.

Our intention is to continue along this dual path since we believe that, if the single approach would not suffice, the combination of the two would. If the SPME capability integrates well with Raman for pheromone detection, it could lead to a new portable and faster tool, and cheaper than GC-MS even if not as sensitive. Furthermore, it could be combined with SERS to increase the sensitivity even further. We intend to pursue that by eluding the volatile adsorbed by the SPME in a vial where the SERS substrate would be exposed, following the approach used in our previous CPRB funded work for the detection of volatile emission profiles in pistachios and almonds [12] which evolution could be associated to fungal growth (aspergilli) as summarized in Figure 2D.

Objective 2 – Use Raman to Measure Pheromone Diffusion

With the Raman detection system in hand, we have been working towards a small-scale pheromone diffusion experiment (i.e. the "the orchard in a box"). The goal is to use an enclosed experimental unit where a few Raman probes are spatially arranged around the central pheromone source, which will then be used to map the concentration and diffusion

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coefficient within the volume of the box as the pheromone is released (Figure 3A). Alternatively, as a first attempt, the lures/dispensers can be arranged in a box containing the Raman gun (Figure 3B) and the environment tested at regular sampling times and at different positions from the Raman gun to investigate the capability to measure the diffusion from the point source. Very initial testing with lures has not been conclusive since the emission rate is very low, while there is also too much leakage, and procedure sampling time and location need improvement. We are currently testing mating disruption dispensers that release pheromones at higher rates. Furthermore, attempts to generate vapors for the semiochemicals are being tried, despite being challenging since the vapor pressure of main chemicals is low at room temperature or high temperature are necessary to evaporate them. Nevertheless, we are considering bubbling toluene at room temperature as it could provide some verification of the detection in space or time and some leads to feasibility. Finally, in parallel, we are building the diffusion models [13] deemed critical to guide the experiments. Once we have more robust results from *Objective 1* on enhanced sensitivity approaches, we will employ them the headspace analysis.

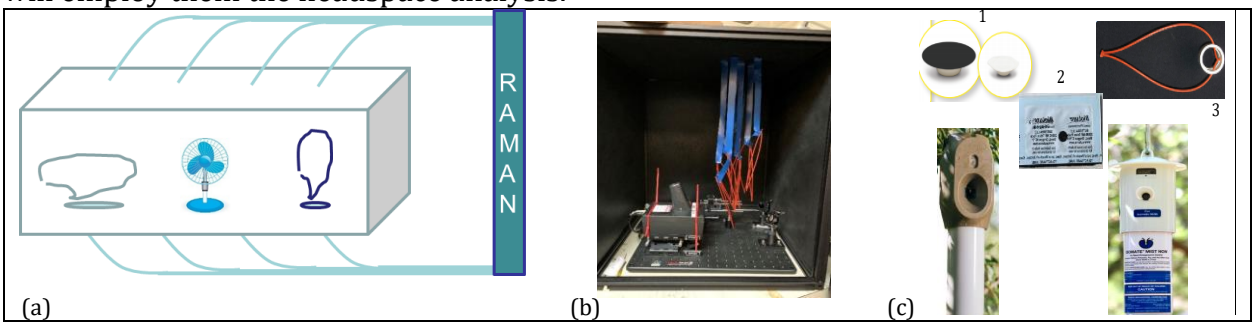

Figure 3. ***(A)*** "Orchard in a box" concept of operation; ***(B)*** initial setups with lures; ***(C)*** future dispensers considered.

In conclusion, with the set of planned experiments, we will validate the enhanced Raman approach as a tool for pheromone emission profiles measurements, a milestone critical for targeting pheromone diffusion measurements in the developing "orchard in a box" experiments, incorporating actual diffusion models. We will have calibration of semiochemical mixtures via PCA also completed for various components mix, that will eventually help deconvolving unknown mixtures. The enhancement by SPME and SERS will be evaluated as well in various configurations, to lead to better diffusion detection setups.
What we are doing here is simply a starting point for a proof-of-concept effort, and we plan to leverage these data into a larger proposal for Federal agencies, to continue the development of this new technology and apply it in orchards. For example, future efforts will Raman coupled to an optical fiber to demonstrate the ability to move around with a nanostructured fiber probe to collect pheromone samples. The fiber would be eventually integrated with the Raman gun for use under field conditions.

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