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Advancing Enhanced Raman Spectroscopy to Detect and Measure NOW Pheromones (CPRB 2022 proposal)

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Disclaimer

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Project Plan / Research Grant Proposal to the California Pistachio Research Board

Workgroup: Pistachios (Pest Management)

Project Year: 2022-2023 (Year 1)

Anticipated Duration of Project: 2 years

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Project Title: Advancing Enhanced Raman Spectroscopy to Detect and Measure NOW Pheromones

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Introduction

Insect sex pheromones are chemical compounds that insects release to attract their partners 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. These pheromone-based strategies can include monitoring, mating disruption, mass trapping, attract-and-kill and push-pull [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 by this pest [4]. Just how mating disruption works is not fully established and likely varies across products and target species [5]. For instance, the extent to which synthetic pheromones compete with natural pheromone is not well understood, or in the case of monitoring, how efficaciously the insect follows the diffusing plumes, especially across large blocks and at plot borders [5]. Furthermore, there may be specific conditions under which poor or impeded diffusion of synthetic pheromone diminishes the disruption effect, which could result in some males effectively locating females for reproduction. At the same time, many commercially available synthetic lures are also available, and growers have been effectively using them to track population development – although the attractive range of these lures remains unclear. That is, while pheromone lures can attract many moths, the relationship between trap capture and local populations, much less crop damage, remains unclear. Regardless of the emission source (aerosol puffers, meso emitters, lures etc.), we currently lack the ability to fully understand how these synthetic pheromone compounds diffuse away from their point-source of emission – and subsequently how this might affect the efficacy of mating disruption and/or the accuracy of monitoring efforts.

Raman spectroscopy (RS) may offer a means to detect synthetic pheromone because of its analytical properties and recent technological advancements [6]. In combination with nanostructured probes, RS has a demonstrated capability to detect volatiles at extremely low concentrations [7]. While other methods for detecting pheromones have proven to be successful, such analytical methods like mass spectrometry (MS) gas chromatography (GC) require sampling in the fields and are not real-time. Other methods such as EAG/ EAD are also invasive (use antennas) but are of course valuable. Opto-electronic, micro and nano sensors just recently enticed interest [8]. We fall in this latter category, proposing the use of optical spectroscopy (Raman) in a fiber aided by nanostructures to provide a chemical fingerprint without transductions and in real-time (i.e. no sampling).

To date, we have been able to generate Raman signatures for the main chemicals in synthetic pheromones (a first for science, to the best of our knowledge), observe their trends at different concentrations, and also provide a clear distinction of such signatures in mixed samples via Principal Component Analysis (PCA) – all of which strengthens our conclusions on the ability of RS to detect emissions in more realistic conditions. We also established processes to enhance the detection sensitivity via preconcentration with Solid Phase Micro Extraction (SPME) fibers and Surface Enhanced Raman Spectroscopy (SERS) to increase the overall optical signals. Furthermore, we explored limitations and requirements for detecting from dispensers, lures, or live moths, including Dept. of Energy and CA State regulatory approvals. Finally, we setup some of our first "orchard in a box" experiments to measure pheromone diffusion along with developing appropriate models.

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In this new funding cycle, we propose expanding our initial work to complete the validation of enhanced Raman Spectroscopy for pheromone emission profiling both in the laboratory and field, as well as provide more quantitative data as outlined in the following sections.

Materials and Methods

We have structured the effort to meet two objectives:

- *Objective 1 – Use enhanced Raman spectroscopy for increased detection sensitivity*
- *Objective 2 - Expand pheromone diffusion experiments in the lab to a field setting*

Objective 1 – Use Enhanced Raman spectroscopy for increased detection sensitivity

In our previous funding cycle, we have been able to generate Raman signatures for the basic pheromone compounds used in pheromone monitoring lures (e.g., Trece L2L). These include: (Z,Z)-11,13-hexadecadienal; (Z,E)-11,13-hexadecadienol; (Z,Z)-11,13-hexadecadienol; and (3Z,6Z,9Z,12Z,15Z)-tricosapentaene. [9,10]. This established a promising baseline for subsequent field studies. We also included (E,Z)-7,9-docedadien-1-yl acetate, the primary compound of the sex pheromone of the European grapevine moth *Lobesia botrana*, that along with the other semiochemicals (provided by Alpha-Scents) was used as a preliminary tests of selectivity. We pursued Raman concentration calibration of the listed components down to a 0.1% concentration in acetone and hexane, which enabled the possibility to evaluate their mixtures (Figure 1A for mix #1). For the latter we have also exploited Principal Component Analysis [11] (PCA) as a proof of concept on how it could help differentiating the signatures (Figure 1B). Principal component analysis is a multivariate method of analysis that helps reducing the dimensionality of a multi–dimensional data set by transforming the original data into a new basis with new coordinates (Principal Components – PCs) that are mutually orthogonal. In Figure 1B we have outlined 3 boxes, the first two (boxes 1-2) identify the single components while box 3 represents the mixtures of the different chemicals diluted in acetone. Each mixed sample falls in the areas between the single chemicals closer to one or another depending on the mix levels. By then focusing on these trends for the same mixture #1 but at different concentrations, we were able to validate the power of the PCA vs simple Raman peak analysis. We also obtained initial results profiling multiple components as detailed in the CPRB 2021 Executive Summary.

For this new proposal, we intend to expand the study mixtures with a larger number of components and at various concentrations that would be more representative of the natural (or synthetic) pheromone emitted profiles. The principal component analysis can then be used to calibrate for the deconvolution of unknown detected profiles.

Having confirmed the validity of Raman detection of the basic semiochemicals, the next goal is to evaluate them in gas phase. Our preliminary test with lures (Pherocon L2L, L2H, Trece) contained in vials were not conclusive because of the extremely low concentration they release and leakages through the vials. We are moving on to mating disruption dispensers since they have 10-100x higher emission rates [CIDETRACK, Trece] of aldehydes at least.

In this proposal, we propose to intensify our conceived new efforts geared towards boosting the sensitivity of Raman with some improvements in the system, specifically,

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by preconcentration of: (1) chemicals via Solid Phase Micro Extraction (SPME) fibers, following the examples with GC-MS [12], and (2) light via Surface Enhanced Raman Spectroscopy (SERS), following examples for other organic volatiles [7]. The two methods are summarized as follows.

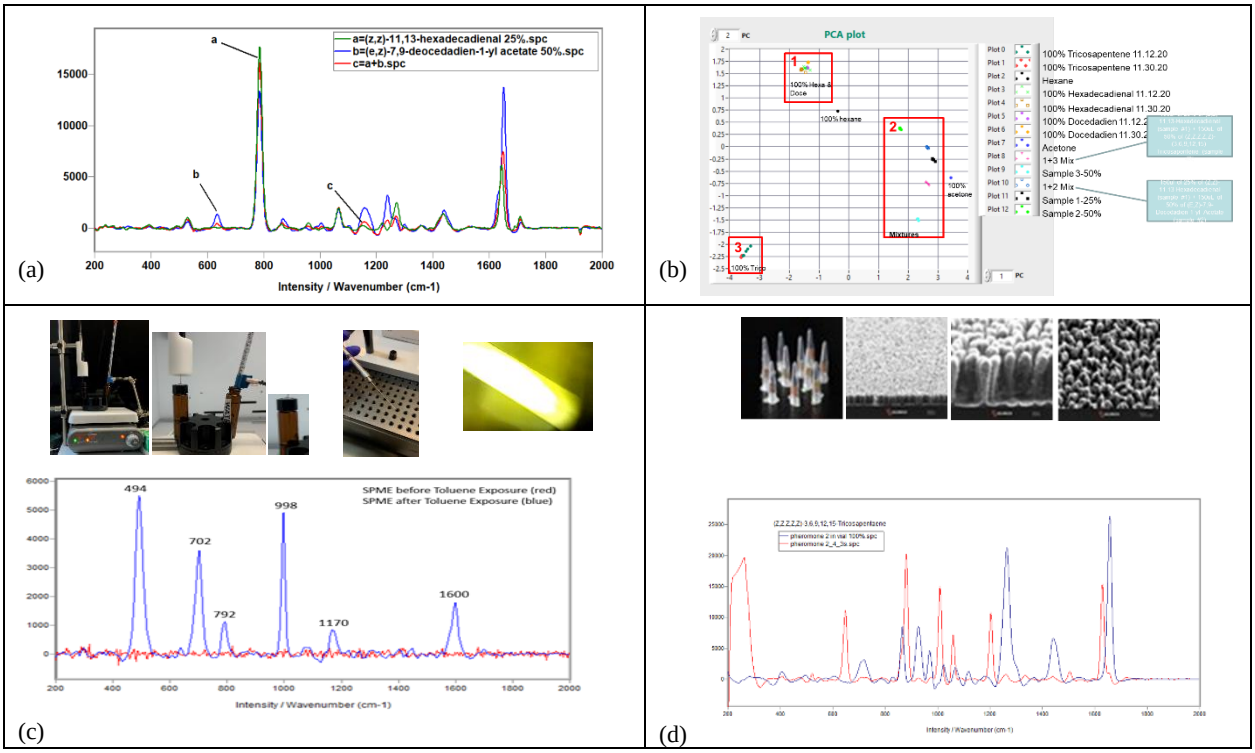

Figure 1. Spectra of mixed samples: **(*A*)** mix #1: 150uL of 25% of (Z,Z)-11,13-Hexadecadienal and 150uL of 50% of (E,Z)-7,9-Deocedadien-1-yl Acetate; **(*B*)** PC1 vs PC2 scoring plot provides between the single components and any mixtures**(C - top)** SPME test setup: fibers exposed just to the headspace of the vial on hotplate, partially filled with toluene; **(c - bottom)** Raman of SPME before (blue plot) and after (red plot) exposure to toluene for 5 minutes at 130C indicating successful uptake; **(D)** (Z,Z,Z,Z,Z)-3,6,9,12,15-Tricosapentaene on Ag substrate.

<u>(Method 1) Preconcentration with SPME.</u>

We have been able to demonstrate uptake in the SPME fibers (50/30um DVB/CAR/PDMS, Stableflex 24Ga from Sigma-Aldrich) and tested them with toluene diluted in acetone solution. The SPME, after being heated for a total of 5 minutes and exposed to toluene in the vial headspace, revealed a successful uptake as shown in the plots of Figure 1C. The second part of the test consisted of interrogating the headspace of an empty vial where the SPME would release the adsorbed toluene, although this did not provide any meaningful results because we could not reach the necessary temperature (>200C as required by GC-MS) to enable the release due to an equipment limitation. We're now updating our setup to enable the headspace release at >200C.

<u>(Method 2) Preconcentration with light.</u>

We have also commenced exploring the use of Surface Enhanced Raman Spectroscopy (SERS) for increasing detection sensitivity. The nanopatterned substrates consist of leaning nanopillars that introduce large number of electromagnetic hotspots which enhance both the light shining onto and scattering back from the molecules. We obtained preliminary

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results after calibration with both bipyridylethylene and toluene as shown in Figure 1D. The observed SERS peaks offer the ability to differentiate compounds from bulk Raman because the vibrational modes are affected by the affinity of the molecules to the unique substrate and how they orientate themselves on it. More studies are required to evaluate the potential of the hotspots in enhancing the signal, but these preliminary results are very promising.

Our intention here is to continue along this dual path, since if the single approach would not suffice, the combination of the two could still possibly produce useable data. The SPME integrated with Raman for pheromone detection would be a useful new tool if sensitivity is combined with high portability, low costs and execution time. In the long term, this 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 as well, or by adding nanoparticles to the SPME itself that would become a SERS probe on its own. SERS of volatile emission profiles for pistachios and almonds was demonstrated by this team before. We investigated signatures selected by USDA that are the most significant markers of growth conditions of* Aspergillus *spp. to provide an early-warning detection signal [13]. The natural path, once we can demonstrate the detection of synthetic pheromones, would be testing for natural pheromones. Having now received approval from USDA and DoE to work with NOW moths in the lab, we have developed a plan to collect NOW adult moths in a vial (from Dr. Wilson) and test their releases via various preconcentrating configurations.

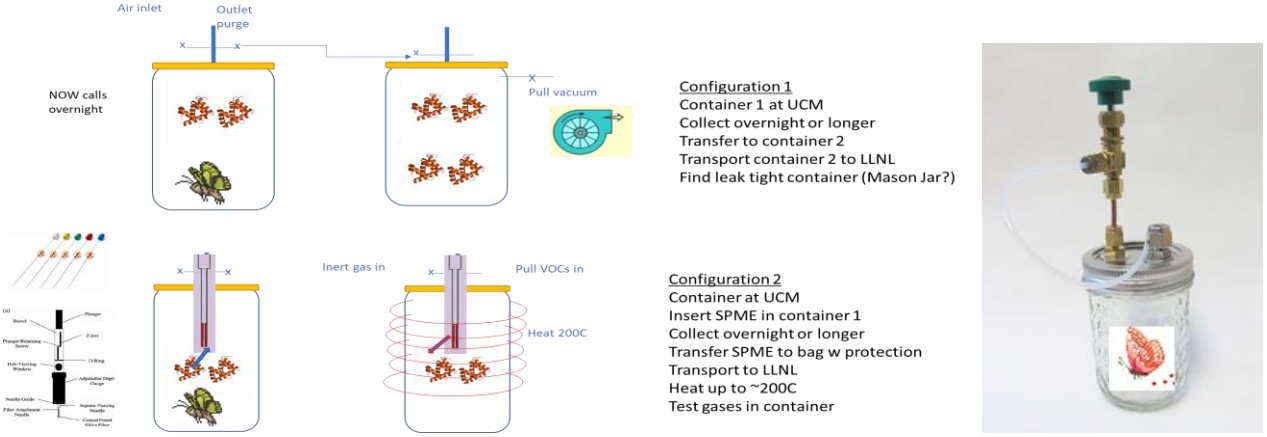

Figure 2. Illustrations of a couple operational schematics being considered for collection of NOW natural pheromones.

Objective 2 - Expand pheromone diffusion experiments in the lab to a field setting

The goal here is to first emulate the orchard and use an enclosed experimental unit (i.e. "orchard in a box") where a few Raman probes are spatially arranged around the central pheromone source, which will then be used to map the concentration and diffusion coefficient within the volume of the box as the pheromone is released (Figure 3A). As preliminary steps, we tried arranging lures in a box containing the Raman gun (Figure 3B), sampling the environment at regular times or at different positions but this has proven inconclusive due to a very low emission rate, vial leakages, and some time/location sampling procedural flaws in the setup. Nevertheless, we have learned a lot from exploring this concept using the commercial products and now understand some of the main limitations.

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We now propose to repeat this set of experiments with mating disruption dispensers (Figure 3C), as they release pheromones at higher rates, then moving on with lures and live moths which have lower emission rates. We will develop baseline experiments via bubbling toluene at room temperature, which will provide some verification of the detection in space or time, and enable optimization of excitation power, signal integration, and spectral resolution. Finally, in parallel, we will boost the development of the diffusion models [14] deemed very critical to guide the experiments. Once we have more robust results with the enhanced sensitivity approaches, we could employ them the headspace analysis. Finally, if time permits, initial testing with Surface Enhanced Raman will be carried out as well.

Using this information, we can begin to understand how pheromone diffusion might work under orchard conditions. The "orchard in a box" experiment is just a starting point to understand pheromone diffusion, and actual diffusion models that incorporate the true complexity of orchard structure and environmental conditions will require significantly more work. What we are doing here is to strengthen a proof-of-concept of direct detection of the pheromones that in the long term might turn to be useful for the community. Under the configuration shown in Figure 3, we can use Raman coupled to an optical fiber to demonstrate the ability to move around with a nanostructured fiber probe to collect pheromone samples. The probes will be eventually integrated with the Raman gun for testing under field conditions and in the fields themselves.

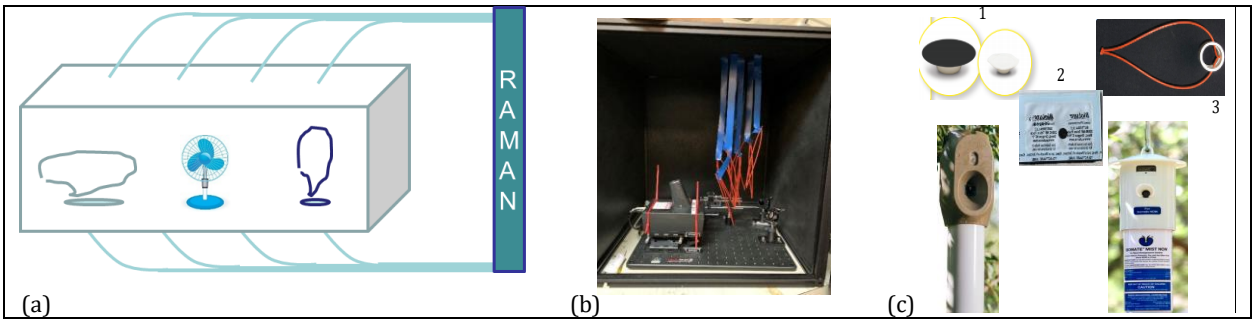

Figure 3A "Orchard in a box" concept of operation; **3B** initial setups with lures; **3C** future dispensers considered.

Projected Results

The goal of this project is to develop the use of Raman spectroscopy to detect synthetic and possibly natural NOW pheromones, which would have a variety of laboratory and field applications to improve our understanding of pheromone diffusion in orchards. Having demonstrated the ability of Raman to detect the primary chemical components of pheromone lures, we will now explore the use of this technology to monitor the diffusion of pheromones within a small, enclosed space. This would be eventually corroborated by basic mathematical diffusion models. In the long-term, these findings will provide the necessary proof-of-concept data to leverage this effort into a larger project funded by Federal agencies (e.g. USDA AFRI etc.)

Contingency Plan

If Raman spectroscopy is still inadequate to detect emission of pheromone from synthetic lures and aerosols, we will investigate alternate additional measurement routes, including (1) measure surface plasmonic resonances vi reflectometry, (2) adding functionalization of

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plasmonic substrates via recognition elements (odor-binding proteins, enzymes, crosslinker polyethylene glycol, aptamers, etc), (3) introduce coated gold nano-tags for sandwiched assays (the nanoparticles could be eventually aerosolized even within a pheromone solution) [8,14], and (2) chemical papers/metals that trap compounds which we can then detect with electrical pulses.

Potential Benefit to California Pistachio Growers

This project will develop the use of Raman spectroscopy to detect synthetic pheromone compounds emitted by trap lures and mating disruption products, as well as natural pheromone emitted from NOW adults. This detection capability will then be used in a basic experiment (i.e. "orchard in a box") to quantify pheromone diffusion in a very stable environment. Preliminary data on pheromone detection will be used to attract additional funding from additional Federal agencies (e.g. USDA AFRI etc.) to fully mature the technology for precision agriculture and pest management applications. The specificity and sensitivity offered by this technique along with being portable, economic, and fast, could lead to the development of a tool for studying the spatial distribution of synthetic NOW pheromone in orchards. Furthermore, it may be possible to use this tool to detect natural NOW pheromone in orchards as well, which would afford some additional research opportunities into the ecology of NOW and behavior in orchards.

Budget Narrative

Funds are requested for (1) a junior scientist to perform Raman analysis for selected pheromones, complete a signature database, design experiments, and perform diffusion analysis, as well as (2) a part-time senior staff scientist to manage the project. Supplies such as basic labware, standards, fibers, substrates, and pheromones will be needed as well.

Budget requested for 2021 (year 2)	**Mar. 2021 – Feb. 2022**
Salary	
Lab Assistant ($1864/month x12 months FTE)	$22,375
Senior scientist ($500/month x 12 months FTE)	$2,527
Indirect Costs	
Lab Assistant – indirect	$25,576
Senior scientist – indirect	$2,900
Supplies	
Labware, lures, aerosols, chemicals, Raman standards, substrates	$5,000
Travel	
Indirect Costs (0%, per agency)	$0
Total	**$59,230**

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