LAWRENCE
LIVERMORE
NATIONAL
LABORATORY

LLNL-TR-740624

Real-time, in-situ detection of volatile profiles for the prevention of aflatoxin fungal contamination in pistachios

T. Bond, A. Chang, J. Zhou

October 25, 2017

Disclaimer

This work performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344.

CPRB 2017 Research Project Progress Report
October 18, 2017

Project Title: *Real-time, in-situ detection of volatile profiles for the prevention of aflatoxin fungal contamination in pistachios*.

Principal Investigator: Dr. Tiziana C. Bond, Dr. Allan Chang, Jenny Zhou - Center of Micro and Nano Technology, Material Engineering Division, Lawrence Livermore National Laboratory, Livermore, CA

Summary

Statement of Work:

The objective in this project is to provide a proof of concept will demonstrate the feasibility of a Raman, in-situ warning system for detecting and removing developing fungal hot spots from pistachio stockpiles and transit containers, thus decreasing human health risks and product loss as a result of contamination. The proposed project has the following goals: to calibrate the Raman fingerprinting of biomarkers, standalone and in premixed samples, to build a database with the vibrational profiles distinctive to the signatures of the bouquet emitted by the contaminated pistachios; to test the improvement in the detection of the detectable markers with enhanced Raman on a small probe.

Tasks, Schedules:

1. Determine the Raman signatures for the most significant components of the bouquet gathered by our USDA collaborator, including contamination at varying humidity levels (FY17-FY18)

2. Calibrate the detection system by comparing standalone biomarker vs controlled premixed samples, with fixed concentrations (FY17-FY18)

3. Evaluate the effect of environment conditions, i.e, humidity, and validate results with USDA collaborator (FY18-FY19).

4. Evaluate Enhanced Raman signal on probe (FY18-FY19)

5. Write Reports (FY18 and FY19)

Progress Report:

The project has practically started in June 2017 when approvals required for Work for Others by DoE were received. The progress reported is thus for the period June-September 2017. We have worked closely with our collaborator, Dr. John Beck at USDA-ARS who has developed volatile emission profiles for pistachios and almonds in different humidity conditions, which evolution could be associated to fungal growth and therefore provide early-warning detection signals (1); similar results were reported by other groups as well (2). We have selected the most significant components of the bouquet gathered by Dr. Beck's team, i.e. the most significant markers of growth conditions, and investigated their Raman signatures. We initially started our measurements with samples provided by Dr. Beck's team and subsequently procured our own. In particular, we have examined isobutyraldehyde, methyl salicylate, ocimene, limonene, pentanal, specifically and also analyzed 5-methyl-furfural, isobutanol, heptanal for almonds as both nuts suffer from similar disease and commonalities could help support any conclusions.

nuts	*main components investigated*
pistachios	methyl salicylate, ocimene , isobutyraldehyde ,*limonene, heptanal*
almonds	5-methyl-furfural, *limonene*, isobutanol, pentanal, *heptanal*

This work was performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344

We have at first taken Raman measurement of all the analytes in liquid phase to retrieve and identify the basic spectrum signature as the signal would be stronger; this was done for each analyte separately. For these measurements we have used a portable Raman system, a Delta-Nu Inspector Raman that was positioned on a stand in our lab for the tests but is designed to be used also as a battery-powered hand-held system for in-situ real-time measurements (Fig.1). Power required for the operation is also very low, (<40mW, although the laser can operate up to 120mW but for lab safety controls we could not go so high- the higher the power the higher the signal and SNR). The wavelength of operation is 785nm which was selected for reducing the fluorescence background. Nevertheless, since it still exists a signal postprocessing software is used to clear the baseline off the signal as shown in Fig 2. Careful selection of the averaging parameters has to be chosen to avoid excluding the signal itself or on the contrary including too much noise. The peaks at specific Raman shifts represent the intensity of the scattered light by the molecules excited rot-vibrational modes.

Figure 1. Intevac Delta0Nu portable Raman system

The cleaned Raman spectrum that we have tested for all the other analytes under investigation are shown in Fig. 3. The measurement s were only 60 secs long. Longer integration time would lead to stronger signal but also stronger noise. The best time is selected after some empirical adjustments.

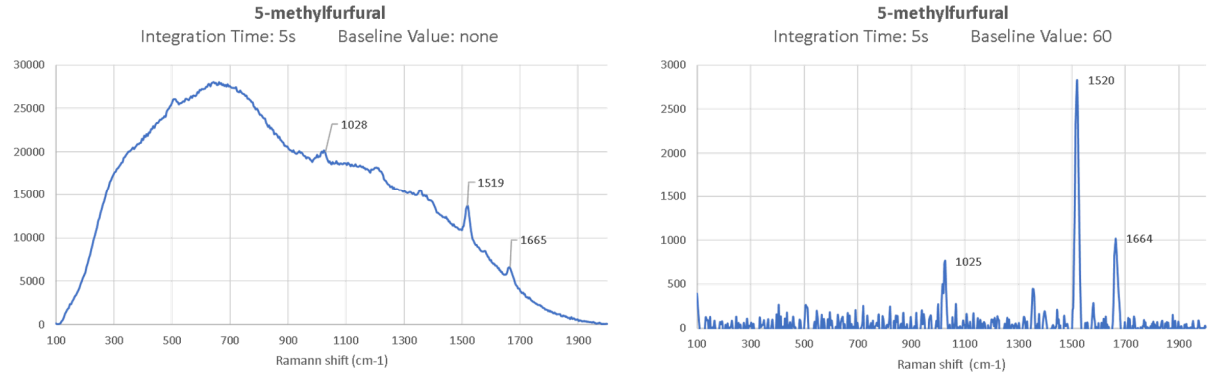


Figure 2. Raman signal for 5methylfirfural with baseline and after baseline is removed. Once the baseline is removed, the peaks are clearly observable and the fluorescence is carefully removed.

This work was performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344

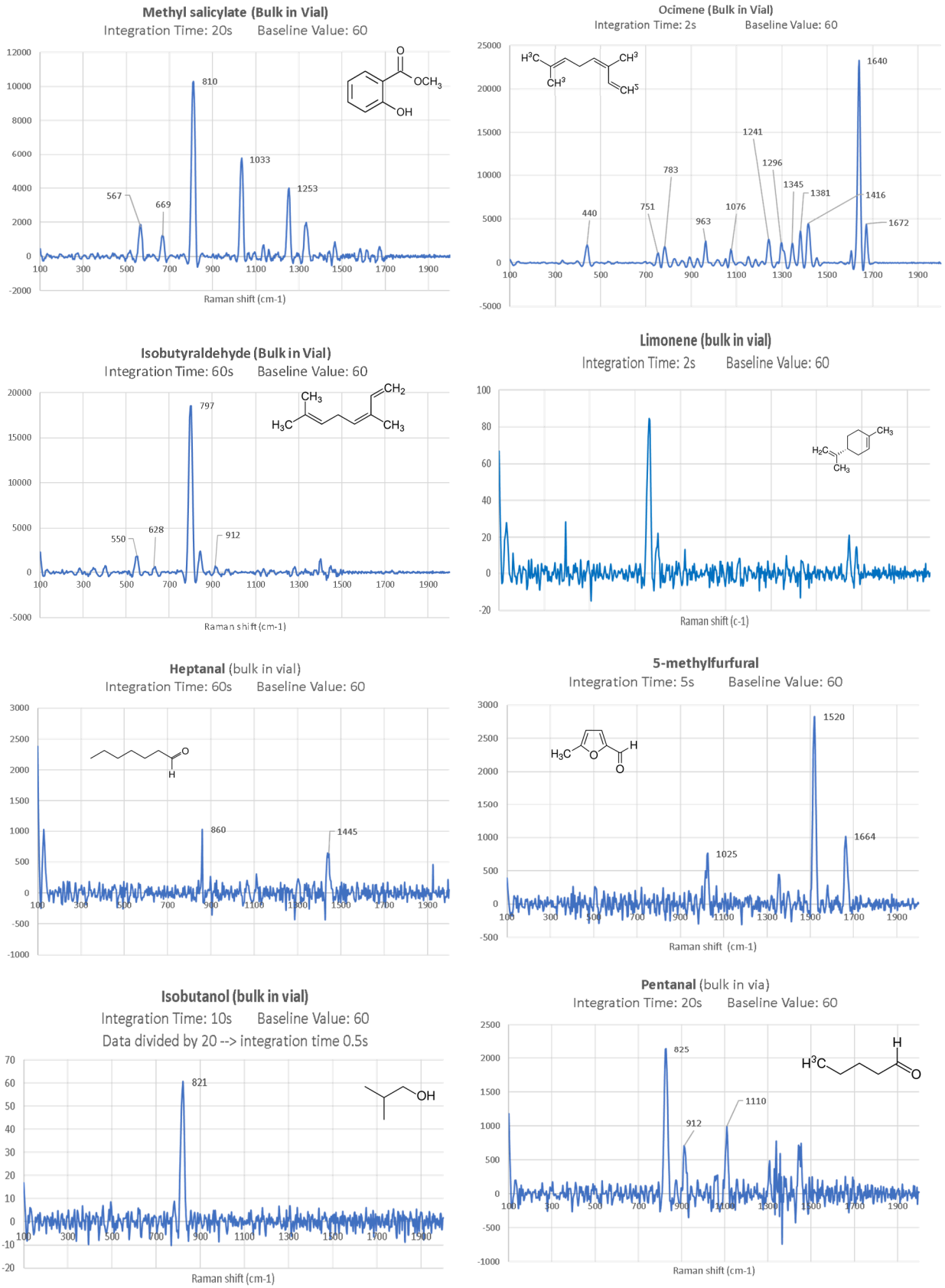
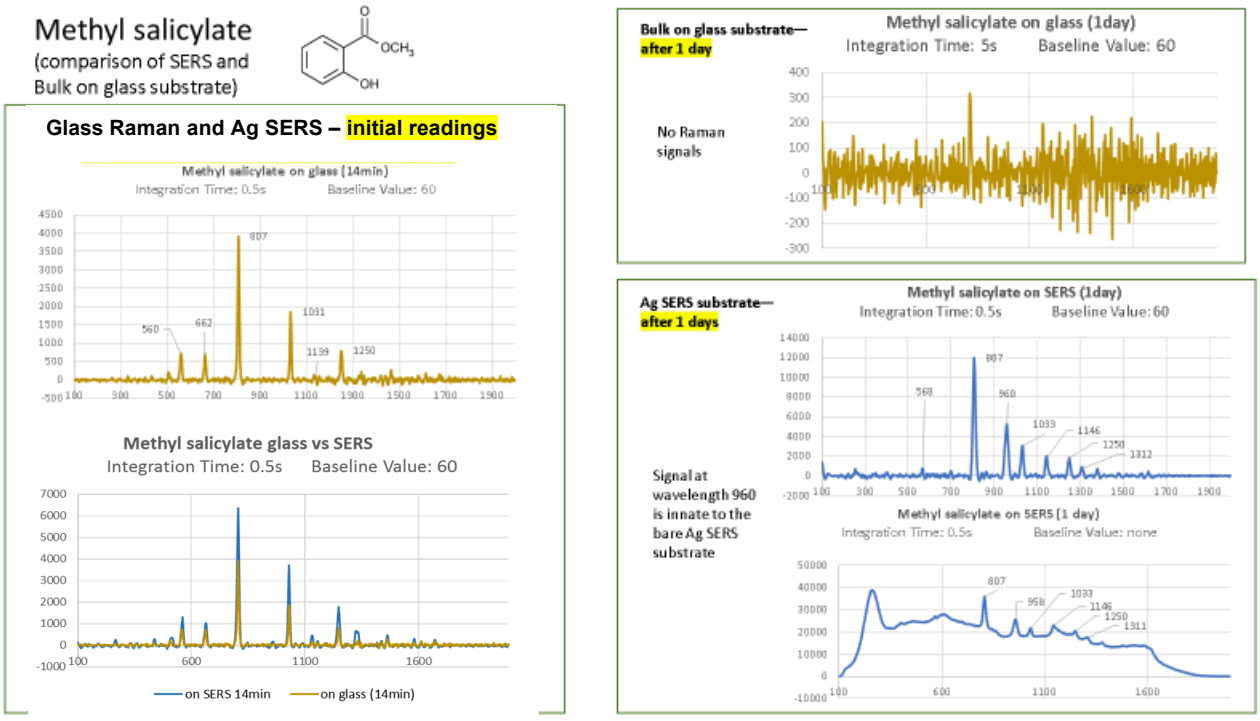


Figure 3. Raman signal for the different analyte of Table I.

This work was performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344

Once we have verified we have signals for the samples under consideration in liquid phase, we proceeded to test 2uL droplets on metallic nanostructured substrates (by Silmeco) for Surface Enhanced Raman Spectroscopy (SERS) signal. These samples provide enhancement of both the light exciting the molecular vibrations at their Raman wavelength and the light scattered back by the molecules (at a slightly shifted wavelength→ therefore we measure the peculiar Raman shift). As an intermediate step we have measured the signal of droplets on the unstructured uncoated side of the slide with the silver or gold nanostructures to have real-time comparison of normal Raman vs SERS. We also systematically characterized the bare nanostructured substrates, just before we cover it with the chemicals droplet, to obtain a background for the SERS signal and avoid misleading readings with the substrate confounding peaks (517cm-1 and 957cm-1). All SERS measurements were between 0.5 and 5 secs long (10x-100x shorter than in normal Raman) and averaged over >5 regions of each Silmeco slide and several repetitions Examples of the set of SERS measurements is shown below in Fig.4. We have taken measurements at various time interval, from hours to days to monitor temporal evolution of the signal. All substrates presented a strong signal after they are exposed to the chemicals ('initial reading') as expected since the droplet provide a large volume contributing to the bulk-like Raman signal. However, we observed some peak intensity increase, most likely to be attributed to the enhancement of the signals from the molecules in proximity of the nanostructure (SERS). This can be actually observed in the bottom left-hand side pictures in Fig.4. With time evolution, we expected to observe a decay in bulk Raman signal due to evaporation or diffusion of the droplet but an initial steady build-up in the SERS signal due to the arrangement of the organic molecules on the surface eventually reaching saturation once the molecules build just a few monolayers.



This work was performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344

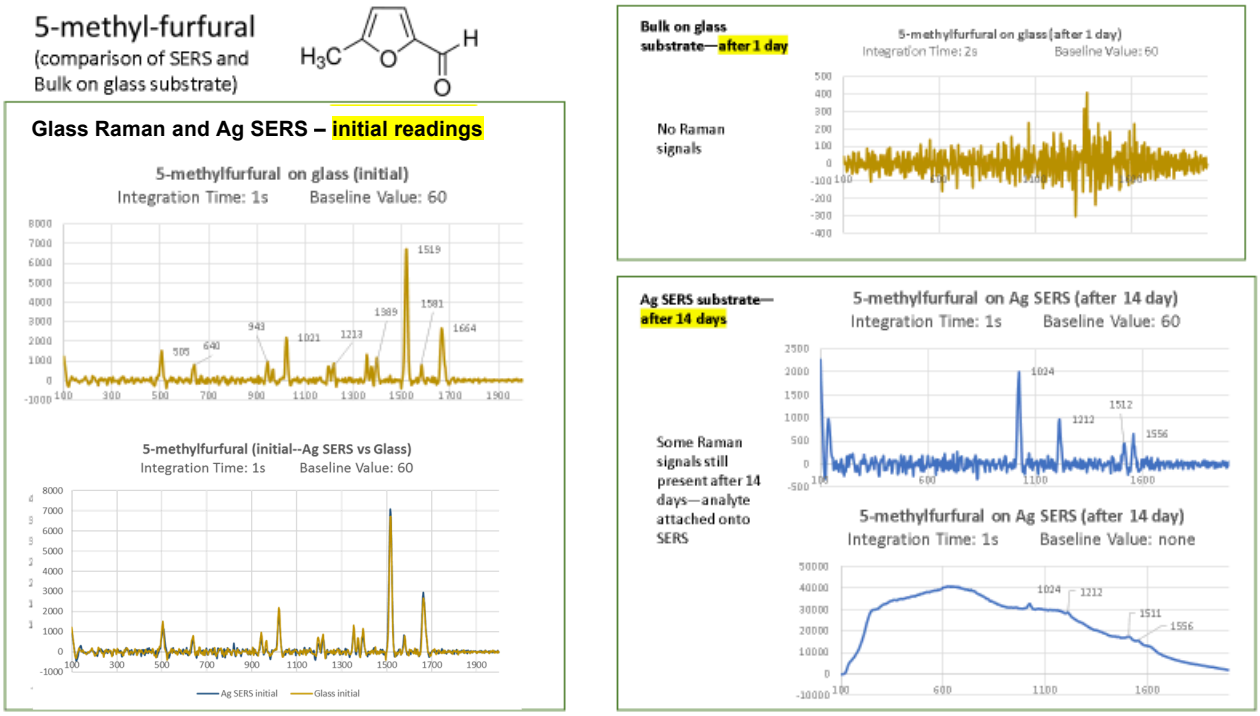


Figure 4. Example of the seconds and third step in the Raman/ SERS measurements on Silmeco slide (glass side vs nanostructure side)

For instance, in the two cases depicted in Fig.4., after one day a 2uL droplet of 5methyfurfural or methysalicylate was dropped on the surface, there is no more significant Raman signal (top right-hand side) but the SERS exists after several days (central right-hand side) indicating that a monolayer had formed and molecules stick to the nanostructured surface enabling a detection otherwise not possible with normal Raman.
Five out of eight chemicals show a strong signal after several days, indicating a strong affinity of the molecules to the substrate and suggesting the potential for gas phase detection. The plots in Fig. 5 show the strength of the signal after several days or hours for methylasalicylate, Ocimne, heptanal (pistachio signatures) and 5-methylfurfaral and isobutylaldehyde (almonds signature). In this set of data, the experimental temporal duration varies because of experimental constraints and scheduling (some samples are still analyzed and therefore show shorter durations). The remaining analytes do not provide any SERS signals almost immediately after the initial reading indicating that either: 1. the equilibrium vapor pressure is very high and therefore tend to evaporate very quickly after being dropped or 2. there is not good affinity between the metallic nanopillars and the specific molecules, or both 1. and 2. We will further investigate these cases since a higher volatility could be beneficial in the gas phase detection. It appears that we can define a retention time for all SERS substrate – we intend to proceed with more systematic study of the temporal evolution with a finer granularity now that we understand how each chemical respond. We hope that we can understand how rapidly the signal changes, saturates and eventually decreases. This is critical information for data interpretation and technology development for any field application.

This work was performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344

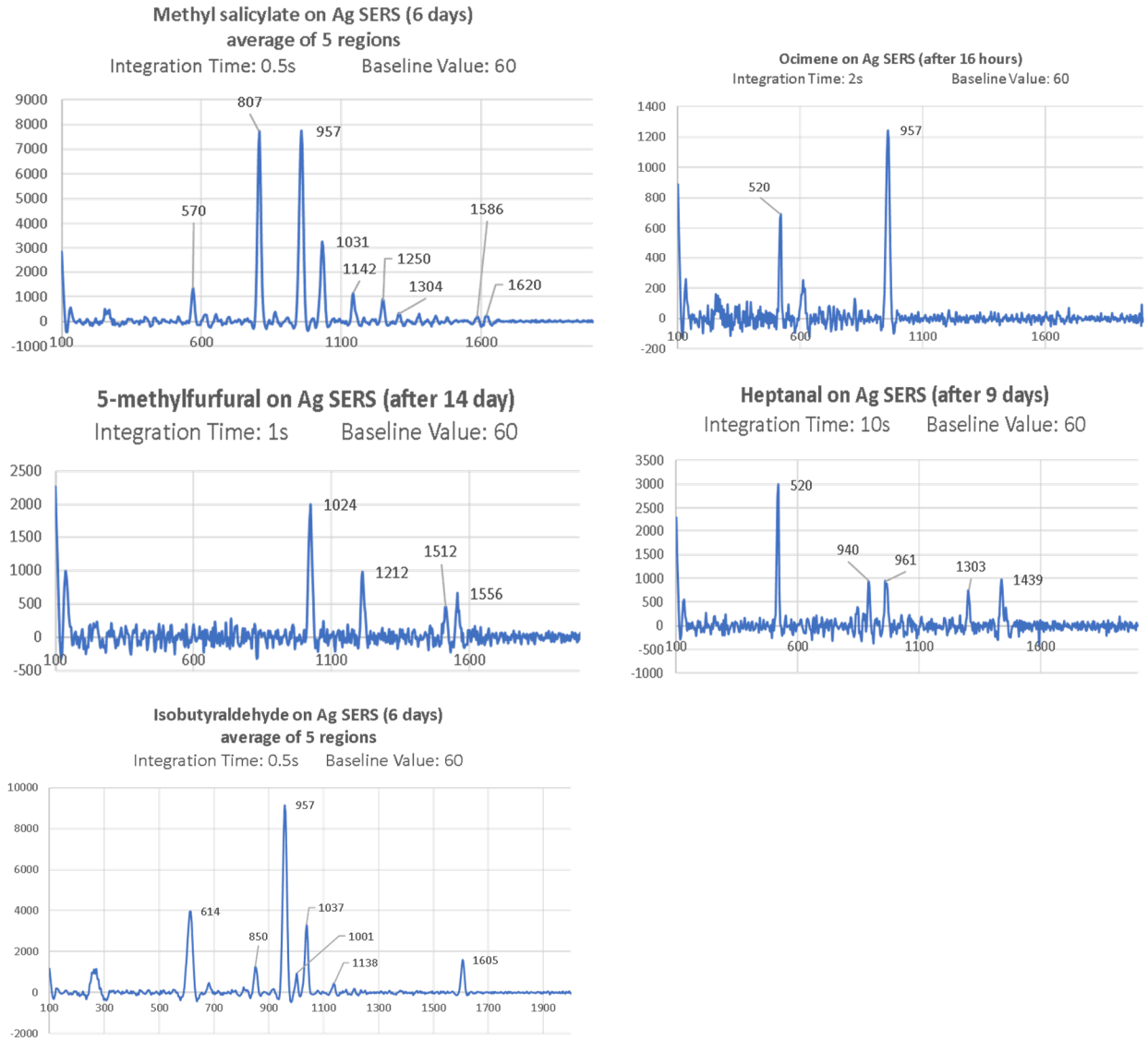
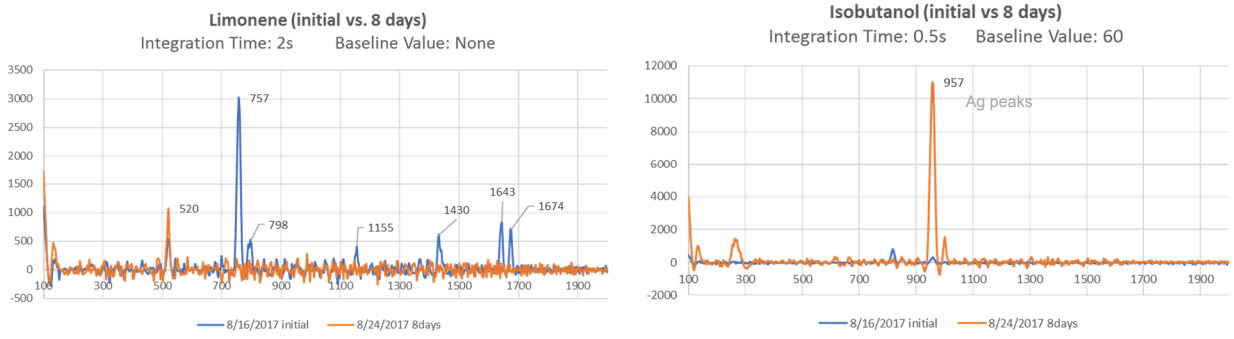


Figure5. Signals for the chemicals that showed a SERS signal several days after the initial readings, indicating stornger affinity to the substrates. Ocimine seems promising but we are comepleting the evelution study.



This work was performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344

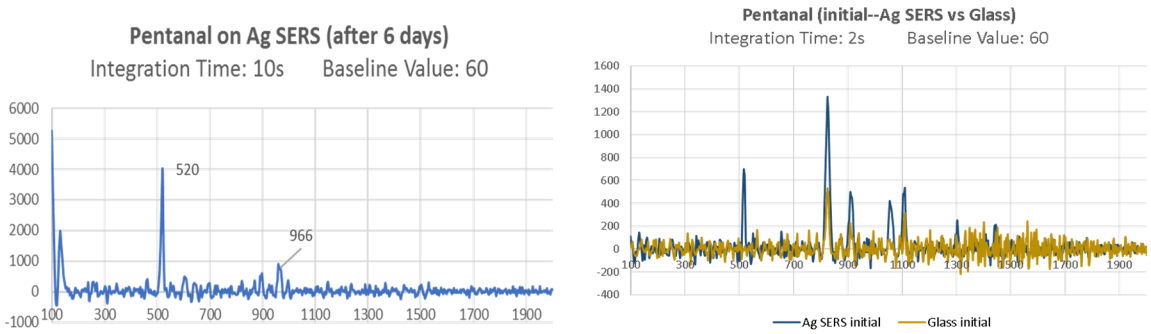


Figure.6 SERS Spectrum for the chemicals that showed **a** faster decay of the signal indicating either less affinity and/or higher volatility

Finally, we have very recently observed an interesting phenomenon. While we were studying two separate samples, one with methylsalicylate and one with 5methylfurfural respectively, it seemed that the latter signal peaks were intermingled with peaks from the former. We immediately attributed this to some evaporation of the methylasalicylate that eventually contaminated the 5methylfurfural substrate. We have therefore launched into a very simple experiment to prove gas-phase detection of the methylsalicylate. We have filled a vial and taped the substrate on its cap so that only the vapor from the analyte could reach the substrate once the vial was capped (Fig.7). The signal appears very strong after 1hr (first measurements) and increases until it reaches some saturation at about 3days and then decays. We postulate that the first hour a partial monolayer is created and then it continues to rearrange, even in multiple layers until the molecules are too far removed from the surface and cannot exploit the enhancement induced by nanostructures. Afterwards, the molecules tend to diffuse away since the adsorption is only based on VanderWaal forces (physisorption) and not covalent ones (chemisorption).

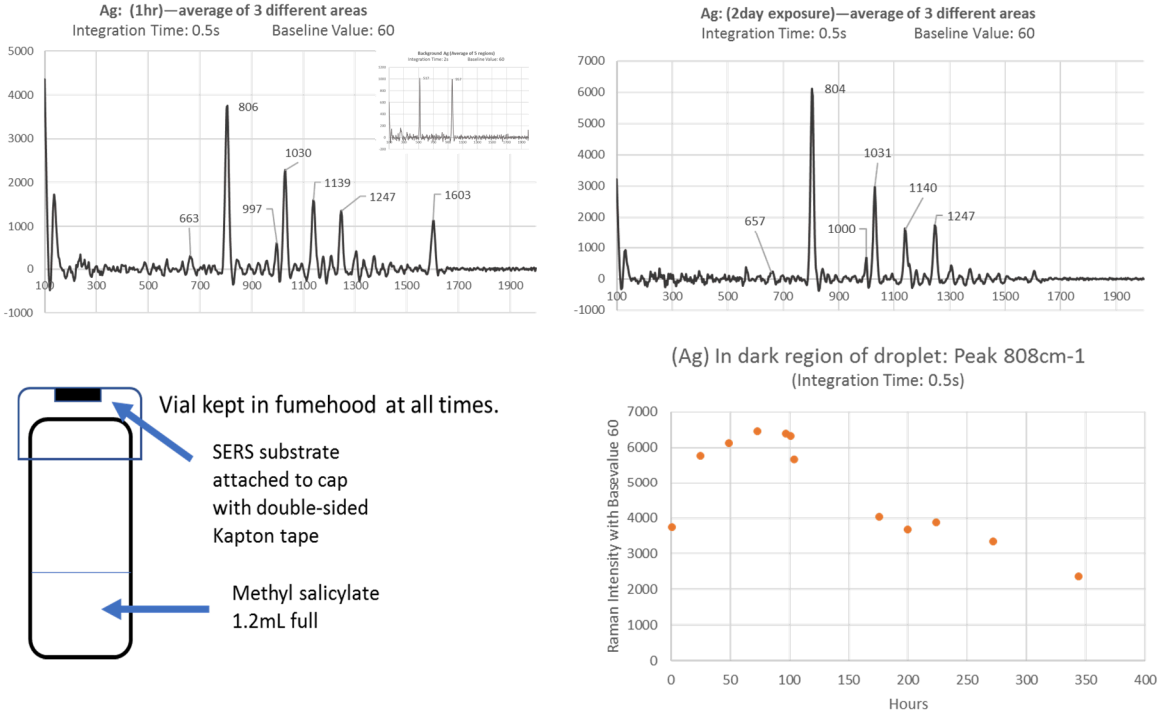


Figure7. Schematic of evaporation experiment ; SERS signal of Ag substrate after 1hr (top left hand side) and 2 days (top right-hand side) exposure to mehtysalicylate l into vial. Inset background of Ag substrate. Plot of 808cm-1 peak in time over 400hrs in the bottom right-hand side, showing initial rising of the signal with some saturation and decrease as expected.

This work was performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344

In conclusion in this first few months of our work we have demonstrate the Raman and SERS signatures of various analytes representative of the pistachios (and almonds) breaths that can also be used as fungi growth markers when related to humidity conditions. For the rest of the funding period we are planning to provide gas-phase analysis for the same group of chemicals. Preliminary results of the detection methylsalicylate vapors provide some confidence on the technique. We will aim at controlling temperature and humidity and provide data with enough spatial and temporal granularity to understand the development of the signal.

Literature

1) Beck et al., Silo-stored pistachios at varying humidity levels produce distinct volatile biomarkders. *J. Agric. Food Chem*. , **2017**, (65), 551-556**.** Beck, J. J.; Mahoney, N. E. Detection of aflatoxins and aflatoxigenic aspergilli in natural fungal bouquets. U.S. Patent No. 9,512,461. Roitman, J. N.; Merrill, G. B.; Beck, J. J. Survey of *ex situ* fruit and leaf volatiles from several *Pistacia* cultivars grown in California. *J. Sci. Food Agric*. **2011**, *91*, (5), 934-942; Beck, J. J.; Willett, D. S.; Gee, W. S.; Mahoney, N. E.; Higbee, B. S. Differentiation of volatile profiles from stockpiled almonds at varying relative humidity levels using benchtop and portable GC-MS. *J. Agric. Food Chem.* **2016**, *64*, 9286-9292.
2) M. Georgiadou , Chr. Gardeli, M. Komaitis , D.I. Tsitsigiannis , E.J. Paplomatas, K. Sotirakoglou , Yanniotis, Volatile profiles of healthy and aflatoxin contaminated pistachiosod Research International 74 (2015) 89–96_

This work was performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344