Bureau of Safety and Environmental Enforcement Oil Spill Preparedness Division

Canine Oil Detection – Using Odor Signatures to Improve Training Detection Proficiency on Land and Water: Final Report

Final Report

October 2021

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US Department of the Interior
Bureau of Safety and Environmental Enforcement
Oil Spill Preparedness Division

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US Department of the Interior
Bureau of Safety and Environmental Enforcement
Oil Spill Preparedness Division

BSEE
Bureau of Safety and Environmental Enforcement

DISCLAIMER

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ABOUT THE COVER

Cover image by Naval Research Laboratory (NRL) showing initial set up with a floating pipe and subsurface mid-level water column delivery.

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From: Commanding Officer, U.S. Naval Research Laboratory
To: Director, Bureau of Safety and Environmental Enforcement (OSRR Eric Miller)

Subj: CANINE OIL DETECTION – USING ODOR SIGNATURES TO IMPROVE TRAINING DETECTION PROFIENCY ON LAND AND WATER YEAR 1 FINAL REPORT

Encl: (1) One copy of subject report

1. Enclosure (1) is forwarded for your information and comment. The purpose of this report is to summarize the work performed during Year 1 of the project entitled *Canine oil detection: Using odor signatures to improve training detecting proficiency on land and water*.

2. The report presents a description of the progress of this program over year, with respect to the objectives and in comparison to the projected milestones and their associated schedule, which include the characterization and comparison of fresh and weathered crude oils, in addition to canine testing to determine the portion of the odor profile of crude oils required for detection.

3. The NRL point of contact is Dr. Steven G. Tuttle, Code 6185, (202) 767-0810.

JOHN N. RUSSELL, JR.
By direction

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CANINE OIL DETECTION – USING ODOR SIGNATURES TO IMPROVE TRAINING DETECTION PROFIENCY ON LAND AND WATER
YEAR 1 FINAL REPORT

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CANINE OIL DETECTION – USING ODOR SIGNATURES TO IMPROVE TRAINING DETECTION PROFIENCY ON LAND AND WATER
Year 1 Final Report

EXECUTIVE SUMMARY

Depending on the severity and length of an oil spill, significant damage to an ecosystem and the economy can occur. A post-Deepwater Horizon study determined that the detection technology for subsurface oil during spill response surveys on shorelines or from pipelines currently relies on techniques that are labor-intensive, have slow survey speeds, and involve only limited or partial areal coverage by spot sampling. This could be obviated by a professional canine detection team that would provide low risk, high confidence support capacity to a survey team. Recent experiences with canines on oil spill response surveys have shown that the oil detection capability of a canine greatly exceeds the requirements of the decision makers who develop specific operational treatment criteria. Although canines have successfully been deployed to detect hidden or obscured crude oil, currently, our advances and developments in the field are typically based on trial and error using the basics of canine detection strategies learned from other applications (mine detection, unexploded ordnance detection, etc.). This research endeavors to elevate the technology to a science-based approach in which we can apply the knowledge gained on the discrimination capabilities of a canine to many real-world situations and to significantly improve training procedures.

Detecting crude oil spills is an important task for environmental quality control; however, it is a difficult task due to the complex vapor profile and effects of degradation. Biodegradation and photodegradation can alter the vapor profile associated with crude oil, which makes it difficult to locate an oil spill if it has seeped into the ground or under frozen water. The aims of this research were to first determine and characterize the odor profiles from fresh crude oils, namely Alaskan North Slope (ANS) and Hoover Offshore Oil Pipeline System (HOOPS), and then determine which portion, or portions, of the odor profiles are used by canines for detection. Furthermore, by exploring the changes in crude odor profiles due to weathering, we can further probe the canine detector capabilities.

Fresh HOOPS crude oil was used to develop and optimize a method of analysis using solid phase microextraction (SPME) with gas chromatography-mass spectrometry (GC-MS). Following method optimization, the odor profile of HOOPS and ANS were characterized and statistically compared. While there were many similarities in the odor profile of the two oils, there were enough differences that they could be statistically discriminated.

Using a Q-SUN Xenon test chamber, fresh crude oil was weathered with irradiation equivalent to the mid-day sun in the southern United States. Weathering produced notable changes in the odor profile of the oil, with the greatest changes occurring in the early-eluting, higher volatility portion of the profile, as well as the addition of poly-cyclic hydrocarbons not seen in the fresh samples.

The chromatograms were separated according to elution order generating fractions of the odor profile that could be captured on a sorbent material and delivered to trained oil detection canines for testing. The canines detected all of the odor profile fractions, as well as the positive controls, with minimal false alerts to blanks or distractors; however, they most readily detected the third fraction containing the heavier semi-volatile compounds. This fraction was also the most similar between types of oil and degree of weathering. The canines were also tested with fractions from the weathered oil. Again, the canines detected all fractions of the oil. The ability of the canines to detect only portions of the oil, fresh or weathered, indicates a deep knowledge of the oil odor and a ready ability to generalize to oils of different type and condition. Future work should be done with canines with more limited experience with crude oil detection, such as canines trained only to fresh oils or only a single type of oil.

Finally, an underwater training prototype device to enable the imprinting and training of canines in the detection of sunken or submerged oils. Canine detection training for underwater targets currently is restricted due to the potential risk of environmental contamination of a water source (lakes, rivers) by training aids that contain oil so that canine teams cannot be exposed to potential oil target odors. The development of the prototype device was intended to address this capability gap. For this purpose, a source of oil is submerged in water in a bucket, outside of the body of water. The headspace, or air above the water, is flushed or bubbled into the body of water, allowing for the odor of submerged oil to enter the water but not the oily contaminants. Prototype and on-shore testing of this device was completed in Year 1.

1 BACKGROUND

"Crude oil" or "petroleum" is the initially retrieved, unrefined mixture of gaseous, liquid, and solid hydrocarbons that deposits in sedimentary rock, accumulating in reservoirs beneath the earth's surface.[1] Crude oil serves as a starting material in the creation of petroleum distillate products such as kerosene, diesel and jet fuel. Over 173 million gallons of crude oil were produced in the US in the year 2020 alone.[2] Ongoing collection, transportation, and processing of crude oil sustains a risk of environmental contamination by crude oil.

Traditional methods of confirming crude oil presence during remediation efforts include the sampling of suspected, contaminated areas for determination of crude oil presence and boundaries of effected zones. These samples are analyzed through multiple techniques including 2-dimensional gas chromatography, 1-dimensional gas chromatography-mass spectrometry, and excitation-emission fluorescence spectroscopy. Of the various approaches utilized, gas chromatography-mass spectrometry (GC-MS) is the standard approach.[3] Due to the structure and

[1] Speight, J. G. *Handbook of Petroleum Refining*, 1st Editio.; CRC Press: Boca Raton, 2016.
[2] U.S. EIA. Petroleum & Other Liquids Report https://www.eia.gov/petroleum/reports.cfm
[3] Christensen, J. H.; Hansen, A. B.; Tomasi, G.; Mortensen, J.; Andersen, O. Integrated Methodology for Forensic Oil Spill Identification. *Environ. Sci. Technol.* **2004**, *38* (10), 2912–2918.

workflow of the laboratory bound portion of the remediation effort, the clean-up procedure can prove to be time consuming, labor intensive, and sometimes futile, as spilled oil can go uncollected during field sampling or be redistributed by natural processes before initial survey results are returned.[4]

In response to these persisting concerns, the use of petroleum detection canines has taken hold, with canines being used to search for surface and subsurface deposits of oil over wide spans of area.[5,6] In a field study conducted by Owens et al. (2017), trained petroleum detection canines (K9-SCAT) were deployed following a residual leak of crude oil from a sunken ship. The K9-SCAT team was deployed 9 months after the residual leak and was imprinted on the "fresh" samples harvested from the source. The canines' team detected visible and non-visible oil deposits at the surface and subsurface level along the shoreline of Chedabucto Bay, Nova Scotia.[6] The positive identification of surface and subsurface level weathered oil deposits suggests a persistence of volatile organic compounds (VOCs) that are indicative of a petroleum product vapor profile.

While the use of petroleum detection canines combats many of the issues presented by a traditionally structured remediation effort, the method that these canines are using to detect crude oil is not fully characterized. The Owens et al. (2017) field study suggests that canines can generalize "fresh oil" to weathered oil. However, to the authors' knowledge, the similarities in the vapor profile of weathered crude oil has not yet been characterized nor applied to understanding the process of canine association of weathered oil samples.

The authors' have constructed a solid-phase microextraction gas chromatography – mass spectrometry (SPME-GC-MS) methodology to characterize "fresh" and weathered crude oil samples, with the aim of understanding which volatile components of crude oil persist through sample weathering. The authors will also use this analysis approach to probe canine detection capabilities and determine which portion, or portions, of the odor profile constitute the odor of interest for canine detection.

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[4] API. Canine Oil Detection (K9-SCAT) Guidelines. *Am. Pet. Institute, Tech. Rep. 1149-4* **2016**, No. July.
[5] Brandvik, P. J.; Buvik, T. Using Dogs to Detect Oil Spills Hidden in Snow and Ice - A New Tool to Detect Oil in Arctic Environments. *Int. Oil Spill Conf. Proc.* **2017**, *2017* (1), 2219–2236.
[6] Owens, E. H.; Dubach, H. C.; Bunker, P.; MacDonald, S.; Yang, Z.; Lambert, P.; LaForest, S. Canine Oil Detection (K9-SCAT) Following 2015 Releases from the T/V Arrow Wreck. *Int. Oil Spill Conf. Proc.* **2017**, *2017* (1), 2620–2641.

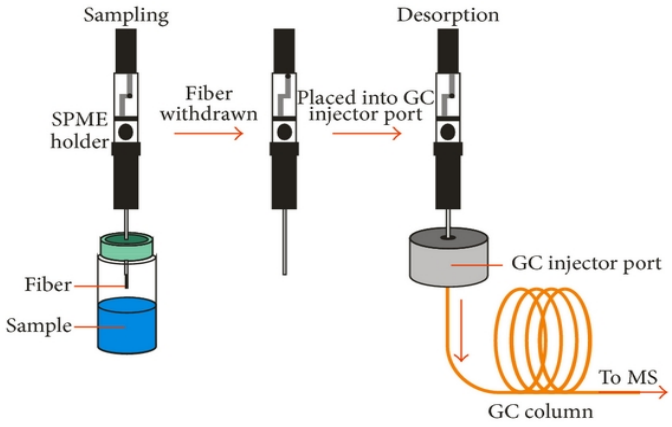
2 RESEARCH TO DATE

2.1 Phase 1 – Headspace Analysis of Aged Crude Oils

2.1.1 Task 1A – Headspace analysis of fresh crude oil

2.1.1.1 SPME-GC-MS Method Development

Headspace analysis was carried out using solid phase microextraction (SPME) with gas chromatography – mass spectrometry (GC-MS). SPME is a non-exhaustive extraction method where a polymer-coated SPME fiber is placed into the headspace of a small amount of crude oil contained in a vial with a septa lid. VOCs from the crude oil adsorb to the polymer coating for a given amount of time. The fiber is then removed from the vial and the analytes are thermally desorbed into a heated injection port in the GC, where the mixture is separated and detected in the MS (Figure 1).



The SPME-GC-MS method was developed and optimized using fresh HOOPS crude oil, starting with a method developed by M. D'Auria, et al. (2008)[7] for the headspace analysis of crude oils, given in Table 1. The D'Auria method resulted in a chromatogram with poor resolution and an unstable baseline. To circumvent these issues, the following parameters were optimized: GC inlet mode (split or splitless), GC oven temperature program, and GC column type. A splitless (100% of sample injected onto the head of the column) was compared to a 10:1 split (10% of sample injected onto column) injection (Figure 2). The split injection resulted in a more stable baseline and overall better chromatogram. Figure 3A shows a chromatogram of the headspace of HOOPS crude oil with at 10:1 split injection and the GC parameters from D'Auria. The resulting chromatogram yielded poor resolution and no compounds eluting after 20 minutes even though the total run time was approximately 38 minutes. After changing the GC column and optimizing

[7] D'Auria, M.; Racioppi, R.; Velluzzi, V. *Journal of Chromatographic Science*, 46(4) 2008, 332-338.

the oven program (Table 1), the peak resolution was improved and the run time was reduced to 35 min (Figure 3B).

Table 1. Starting and final GC parameters for the headspace analysis of crude oil.

Method Parameter	*Starting*	*Final*
Inlet mode	Splitless	10:1 split
Oven program	40 °C, hold 2 min	40 °C, hold 5 min
	40-250 °C at 8 °C/min, hold 10 min	40-80 °C at 8 °C/min
		80-200 °C at 5 °Cmin, hold 0.5 min
GC column	ZB-5 MS (Phenomenex)	RTX-Volatiles (Restek)

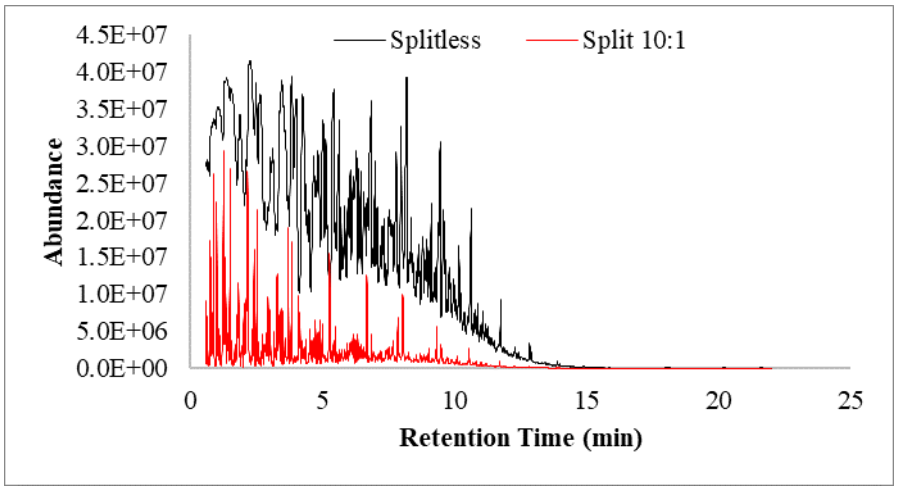


Figure 2. Chromatograms of HOOPS crude oil resulting from a splitless (black trace) vs. 10:1 split (red trace) injections.

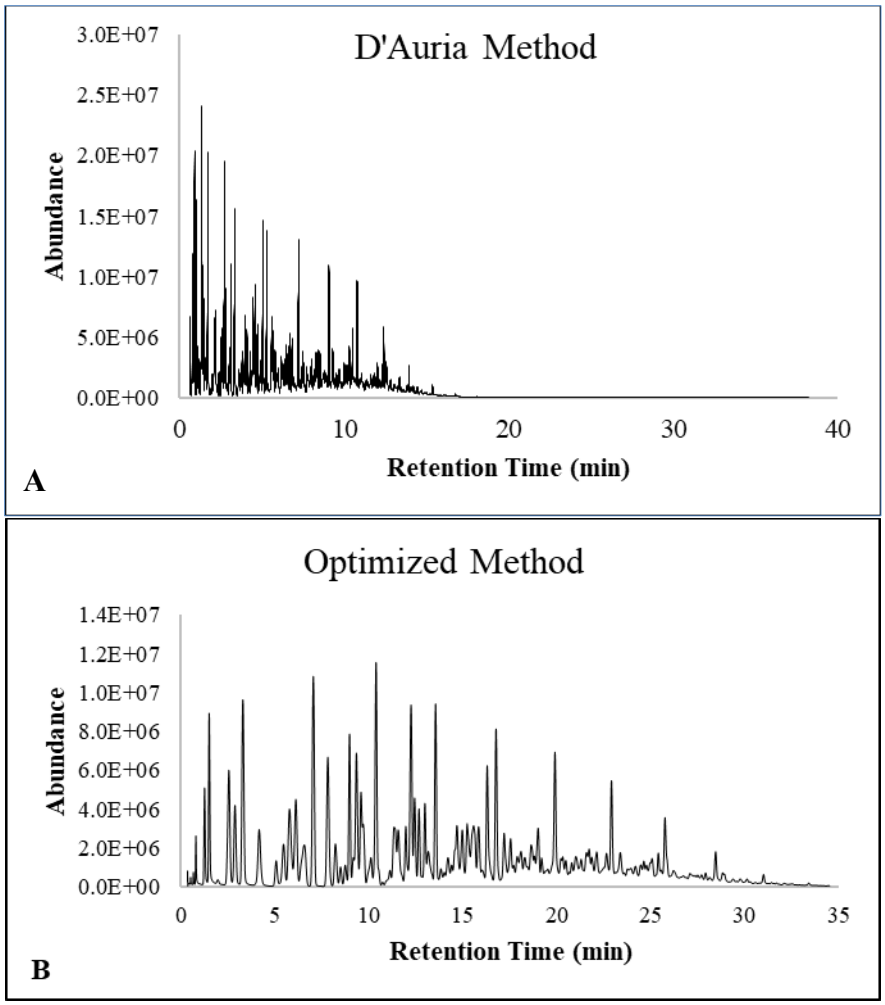
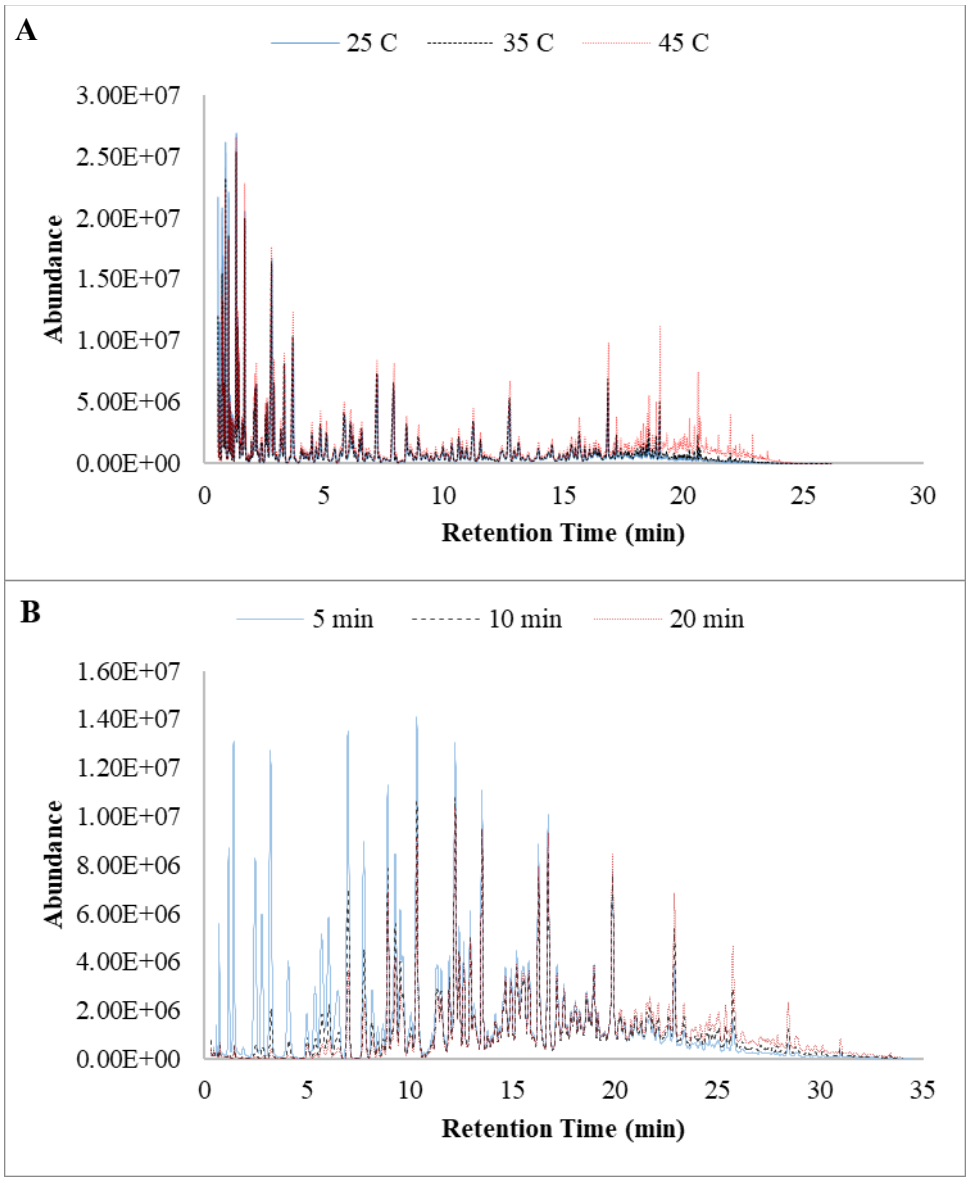


Figure 3. Chromatograms from the headspace of HOOPS crude oil using GC parameters from M. D'Auria, et al. (2008) (A) and final optimized method (B).

The following SPME parameters were optimized: SPME extraction temperature, SPME extraction time, sample equilibration time, and SPME fiber type. Three extraction temperatures were investigated in triplicates: 25 °C, 35 °C, and 45 °C, with an equilibration time of 0 minutes and an extraction time of 20 minutes. Extraction temperatures on the lower and higher end resulted in loss of long chain hydrocarbons and short chain hydrocarbons, respectively (Fig. 4A). Thus, to preserve the presence of both short and long chain hydrocarbons, a moderate temperature of 35 °C was selected. Using an extraction temperature of 35 °C and equilibration time of 0 minutes, three extraction times were examined in triplicates: 5, 10, and 20 minutes. Extraction time of 5 minutes did not allow for longer chain hydrocarbons to adsorb onto the fiber, and while an extraction time of 20 minutes allowed for adsorption of the long chain hydrocarbons reduced extraction of short chain hydrocarbons due to competition for binding spots on the polymer fiber occurred (Fig. 4B). To achieve adsorption of both short and long chain hydrocarbons onto the SPME fiber, an extraction time of 10 minutes was selected. Three equilibration times were investigated in triplicates: 0, 10, and 20 minutes, using an extraction temperature of 35 °C and extraction time of 10 minutes (Fig. 4C). Ultimately, an equilibration time of 20 minutes resulted in the presence of

more hydrocarbons than 0 or 10 minutes equilibration, and longer equilibration times did not change the outcome; thus, 20 minutes was selected. Lastly, using the optimized SPME parameters, four types of SPME fibers were considered: Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS), CAR/PDMS, PDMS/DVB, and 100 µm PDMS (Fig. 4D). The 100 µm PDMS fiber yielded the greatest abundance of VOCs onto the fiber as compared to the other fibers. Final SPME parameters are listed in Table 2.


A
25 C
35 C
45 C
3.00E+07
2.50E+07
2.00E+07
1.50E+07
1.00E+07
5.00E+06
0.00E+00
Abundance
0
5
10
15
20
25
30
Retention Time (min)
B
5 min
10 min
20 min
1.60E+07
1.40E+07
1.20E+07
1.00E+07
8.00E+06
6.00E+06
4.00E+06
2.00E+06
0.00E+00
Abundance
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25
30
35
Retention Time (min)

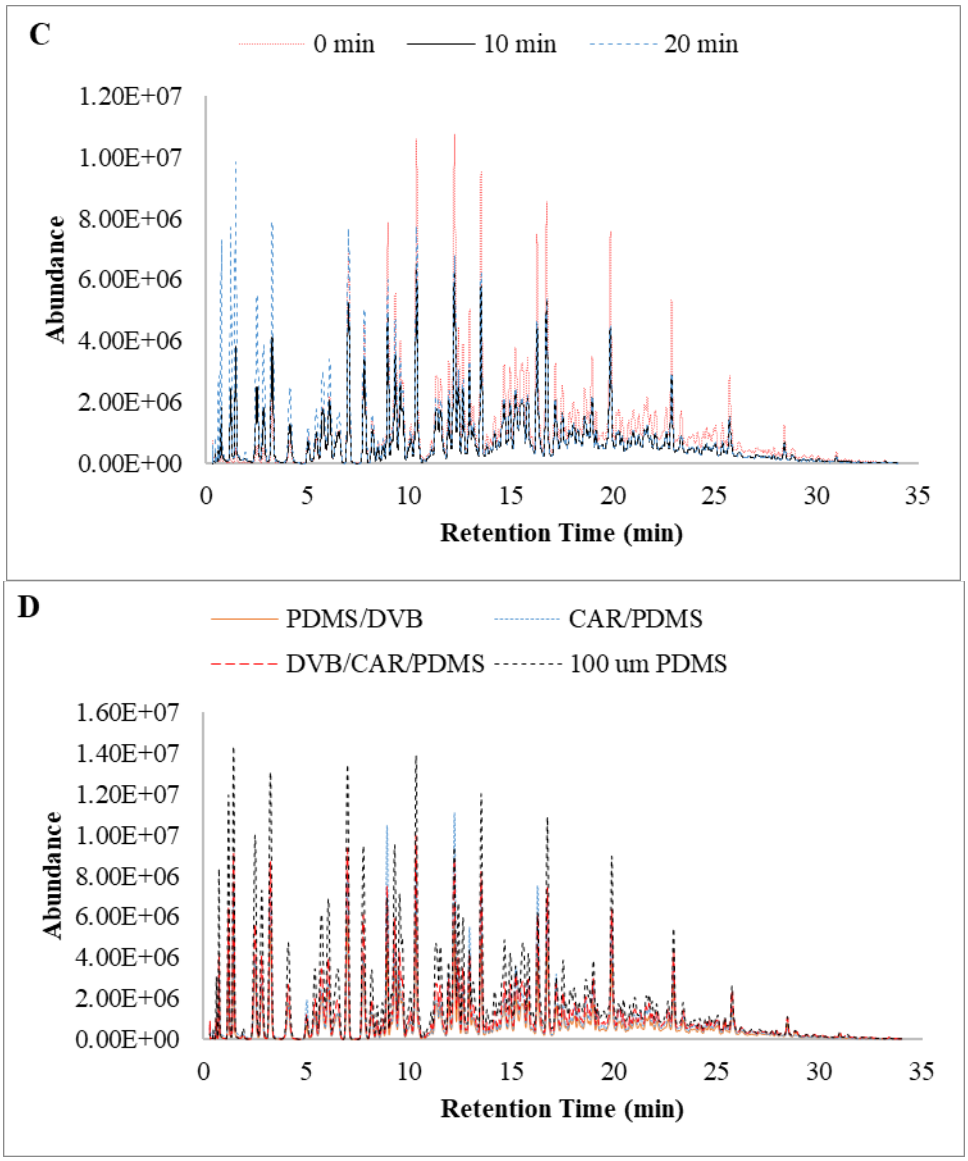

Figure 4. Chromatograms from the headspace of HOOPS crude oil for optimization of SPME parameters: extraction temperature (A), extraction time (B), equilibration time (C), and fiber type (D).

Table 2. Equilibration and SPME parameters.

Final SPME Parameters	
Extraction Temperature	35 °C
Equilibration Time	20 min
Extraction Time	10 min
SPME Fiber	100 µm PDMS

2.1.1.2 Headspace Analysis of Crude Oils

The headspace analysis of HOOPS and ANS was accomplished using the SPME-GC-MS method. The headspaces of HOOPS and ANS contained a total of 68 peaks each, which corresponded to a predominate number of alkanes, cyclic alkanes, and aromatic compounds. In Figure 5, the VOCs

extracted from the headspaces of HOOPS and ANS were classified by respective functional groups. Here, it can be seen that the odor profiles of HOOPS and ANS slightly differed, with HOOPS being composed mainly of branched and straight alkanes, followed by aromatics, cyclic alkanes, and then terpenes and polycyclic aromatics. Comparatively, ANS could be differentiated by an increased abundance of cyclic alkanes and reduced quantity of branched alkanes (Fig. 5).

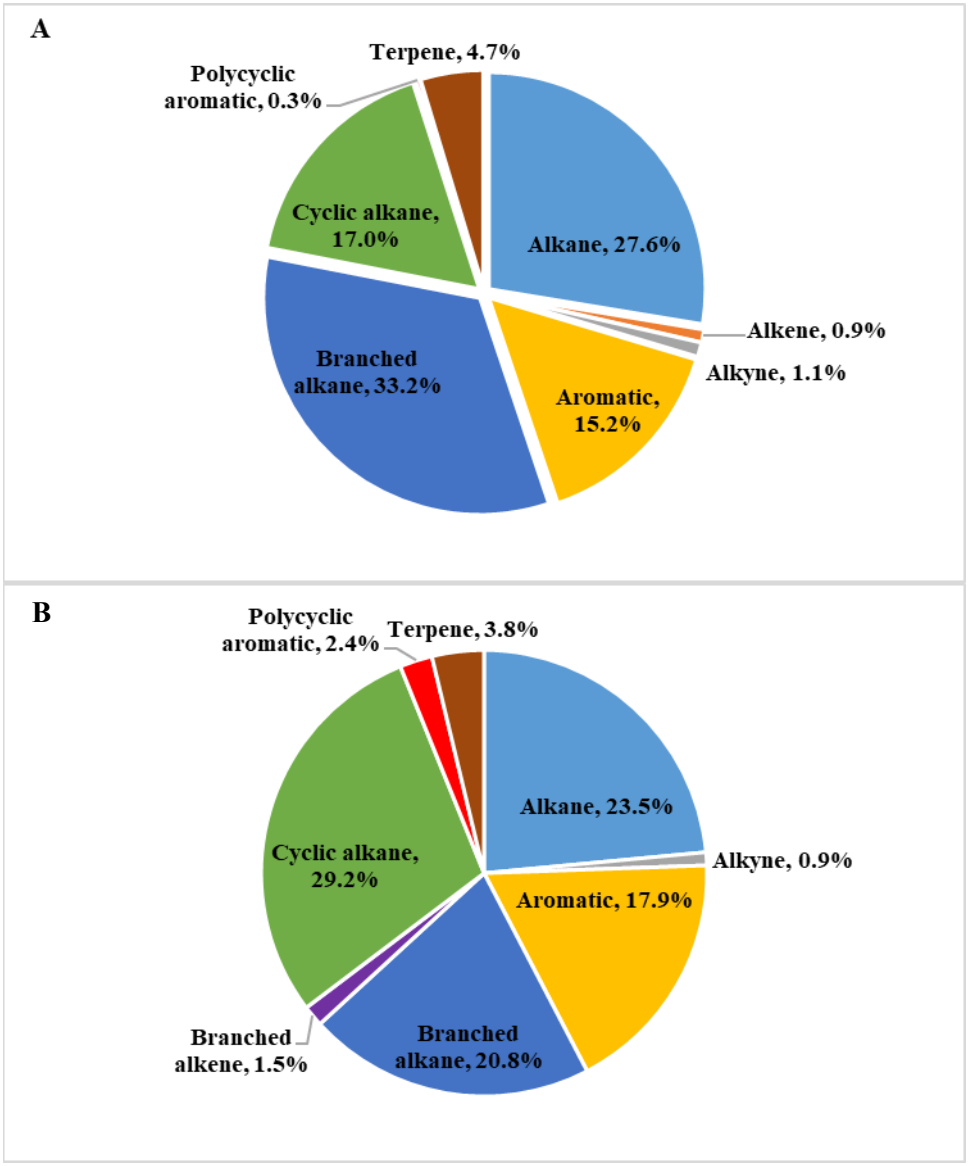

Figure 5. Compound class distribution of VOCs extracted from the headspace of HOOPS (A) and ANS (B).

2.1.1.3 Data Handling and Statistics Technical Approach

To statistically compare the similarity between the odor profiles of HOOPS and ANS, a chemometric approach was designed for analysis of complex chromatograms resulting from the crude oils. The intra-sample and inter-sample variations in crude oil headspaces were quantified using Spearman's rank correlation, dynamic time warping, and 3-D covariance. Spearman's rank correlation and dynamic time warping respectively yield correlation coefficients and similarity

measures that relate to the comparison of total ion chromatogram (TIC) features between samples. 3-dimensional (3-D) covariance relays a similarity measure that is representative of compositional differences in compared profiles. Aggregated data was analyzed using supervised and unsupervised machine learning algorithms, including principal component analysis (PCA), linear discriminant analysis (LDA), and hierarchical clustering analysis (HCA).

The planned approach for chemometric analysis of the headspace profile of crude oils required peak alignment across the dataset. A software program was developed to perform the peak alignment procedure across a submitted dataset (i.e., chromatogram data). Additionally, programs for the conductance of dynamic time warping and 3-D covariance were developed to perform data preparation alongside the chemometric analysis.

In preparation for the implementation of the planned chemometric approach for analysis of headspace volatiles from crude oil samples, necessary data software programs were developed and refined. The developed programs performed the functions of (1) peak matching, (2) 3-D covariance calculation, and (3) dynamic time warping. Program 1 – Peak Matching, performs retention time-based peak matching across multiple data files. This program corrects for retention shifts viewed in compound confirmed peaks. Additionally, the program performs alignments between samples collected on GC columns of differing lengths. Due to the untargeted nature of the performed SPME-GC-MS method, this program acts as a data preparation step for Spearman's rank correlation, PCA, LDA, and HCA identifying peaks of interest and their appearance across the submitted sample set.

Program 2 – 3-D Covariance calculation, performs data retrieval, denoising, and the procedure for covariance calculation. 3-D covariance determination allows for the assessment of a measure of similarity between 3-dimensional data output from GC-MS data files. The program performs data preparation as well as a pairwise 3-D covariance calculation between all submitted files.

Lastly, Program 3 – Dynamic Time Warping, also performs data retrieval, denoising, normalization, and the dynamic time warping function. It computes the stretch or warping in the time axis that allows for the optimal alignment of two time series. The cumulative distance between aligned elements can be expressed as the cost of the path. This program allows the user to perform a pairwise determination of cost of path between all submitted sample files. The determined cost of path acts as a similarity measure indicating the degree of TIC alignment between tested samples.

In all, these software programs were used in the chemometric analysis comparing crude oil headspace samples. In its application, the devised approach will inform a greater body of work regarding the volatile components of crude oil, how these components change when subjected to weathering, and how separately sourced crude oil samples vary throughout this process. These findings will garner fundamental understanding of crude oil composition and weathering, informing crude oil detection procedures.

2.1.1.4 Chemometric Analysis of ANS and HOOPS

Peak alignment and matching were completed in R[8], using a peak bin width of 0.040 minutes and a peak height threshold of 1,000 (Abundance Units). Following an initial peak matching procedure, the results were filtered for peaks present in all three triplicates of the corresponding sample. In total, 68 peaks met these criteria. 63 peaks were present in all triplicates of ANS, 62 peaks in all triplicates of HOOPS, and 55 of those peaks were present in all triplicates of both samples.

Figure 6. Venn diagram of retention time matched peaks.

The curated list of retention time-matched peaks was submitted to a 2-tailed t-test (with equal variance assumed) in order to determine which peaks were statistically significantly different in terms of TIC peak areas. Of the submitted dataset, 42 peaks were found not to be statistically significantly different ($p \geq 0.05$), while 26 peaks were found to be statistically significantly different ($p < 0.05$). These compounds are indicative of sample similarities that may be true to the category of fresh crude oils.

The common peaks (in both name and quantity) between HOOPS and ANS are shown in Figure 7. In this bar graph, each color is a different compound and the size of the color bar equates to the proportion of that compound in the headspace. From Figure 7, one can see clear similarities between the odor profiles of ANS and HOOPS.

[8] The R Project for Statistical Computing; r-project.org

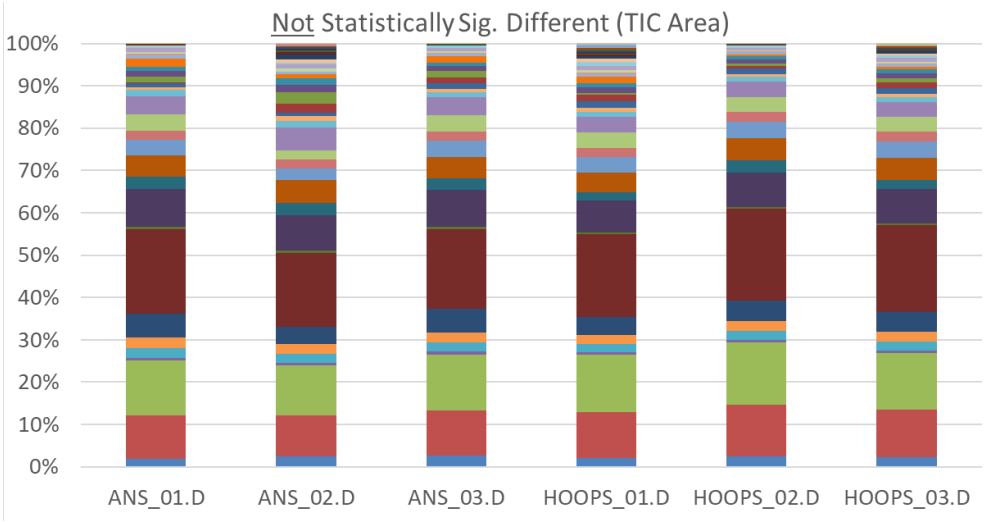

Figure 7. Headspace components present in statistically similar quantities between triplicate samples of ANS and HOOPS.

It is possible to use the remaining volatiles to differentiate between the odor profiles of ANS and HOOPS. Figures 8 and 9 compare the compounds that are present in only one of the crude oils or in statistically different quantities.

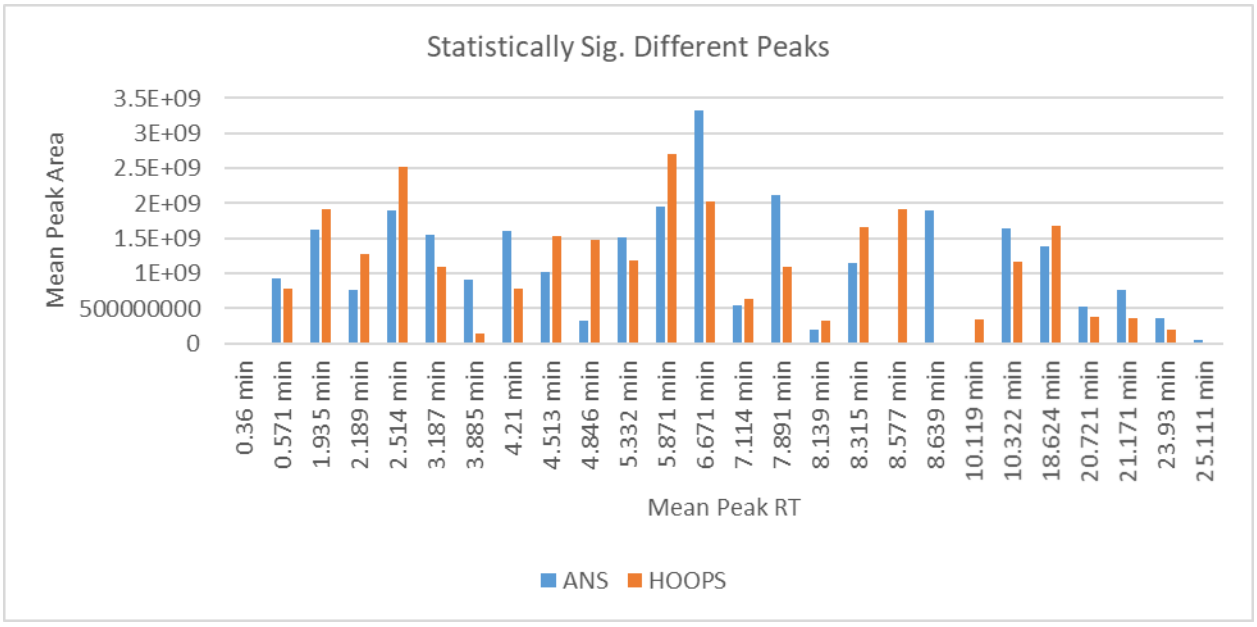

Figure 8. Mean peak area of compounds found to be statistically different between ANS and HOOPS oils.

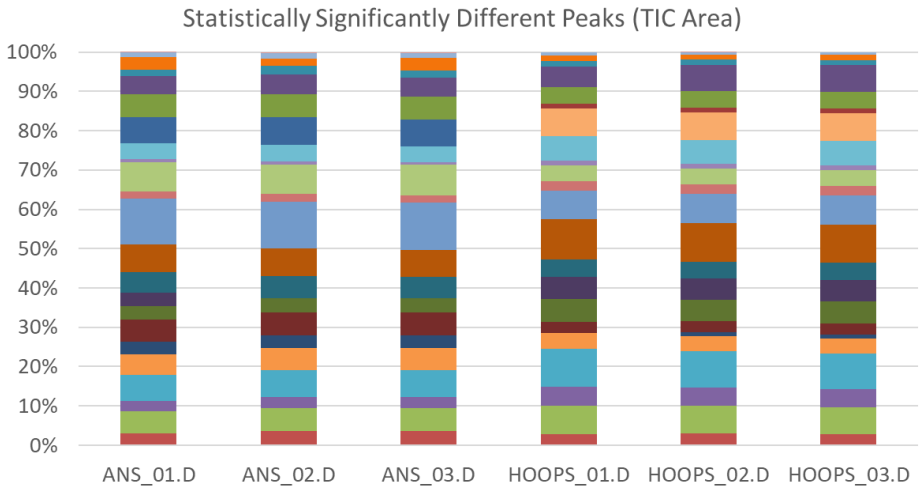

Figure 9. Headspace components present in statistically different quantities between triplicate samples of ANS and HOOPS.

Spearman's rank correlation was performed using the peak areas of retention time-matched peaks. The values displayed in Table 3 are the Spearman's rank correlation coefficients (Spearman's rho or ρ); a higher value denotes a stronger correlation in the information being compared. Table 3 is colored in accordance with the optimal performance level of Spearman's rank correlation test (SR). Optimal performance occurs when implementing the following thresholds (1) Association $\rho \geq 0.80$, (2) Dissociation $\rho < 0.80$ (ρ = Spearman's coefficient). The green values have been associated; the uncolored values have not been associated.

Table 3: Spearman's Rank Correlation (ρ)

	ANS_01.D	ANS_02.D	ANS_03.D	HOOPS_01.D	HOOPS_02.D	HOOPS_03.D
ANS_01.D	1.000	0.923	0.945	0.752	0.793	0.786
ANS_02.D		1.000	0.979	0.688	0.727	0.726
ANS_03.D			1.000	0.701	0.746	0.738
HOOPS_01.D				1.000	0.967	0.974
HOOPS_02.D					1.000	0.992
HOOPS_03.D						1.000

3-D covariance calculations were performed using the unabridged 3-D dataset from the whole chromatogram, as opposed to the individual peaks as was done in the Spearman rank correlation. The value displayed is a similarity value; a higher similarity value (S) indicates that the compared samples are more similar than a set with a smaller value. Table 4 is colored in accordance with the optimal performance level of the 3-D covariance test. Optimal performance occurs when implementing the following thresholds (1) Association $S \geq 0.85$, (2) Dissociation $S < 0.85$ (S = similarity value). The green values have been associated; the uncolored values have not been associated.

Table 4: Dynamic Time Warping (γ).

	ANS_01.D	ANS_02.D	ANS_03.D	HOOPS_01.D	HOOPS_02.D	HOOPS_03.D
ANS_01.D	0.000	61.764	20.907	115.668	110.839	110.943
ANS_02.D		0.000	68.192	145.910	139.005	139.984
ANS_03.D			0.000	113.164	107.007	106.623
HOOPS_01.D				0.000	20.731	17.517
HOOPS_02.D					0.000	17.703
HOOPS_03.D						0.000

Note that all of the triplicate sets have matched to one another, while all HOOPS and ANS samples have been differentiated using both statistical tests.

2.1.2 Task 1B – Headspace Analysis of Weathered Crude Oil

2.1.2.1 Simulated Weathering of HOOPS

In Quarter 3, NRL received a Q-Sun Xe-3 Xenon test chamber (Q-Lab) for the simulated weathering of HOOPS crude oil (Fig. 10A). Briefly, approximately 1 g of fresh HOOPS crude oil was placed in a Pyrex petri dish and irradiated for 12 hrs at an irradiance of 0.68 W/m^2 at 340 nm. This irradiance was selected as it is approximately equivalent to the summer sun in the Southern U.S. at noon. Following irradiation, the petri dish was placed in a Teflon jar with septa in place in the lid for SPME sampling, followed by GC-MS analysis. The SPME-GC-MS method used was the same as described above in section 2.1.1.1. To better understand how the odor profile changes over time when exposed to the "sun", multiple irradiation times were investigated: 4, 24, and 168 hours. All samples were prepared in triplicate.

Figure 10B and C visibly compare the change in appearance of the oil from fresh to 12 hours of irradiation. Figure 11 shows the chromatograms produced from the headspace of fresh and weathered crude oil. After only four hours of irradiation a significant change in the odor signature can be seen, with the total abundance of VOCs decreasing substantially and the majority of the high volatility compounds (HVOCs), eluting before 11 min, no longer being detected. There was little change from 4 to 12 hours of weathering. After 24 hours, there was a greater reduction in total abundance and the few compounds eluting before 18 min could be detected. Finally, after 168 hours of irradiance, there were only a small number of the semi-volatile compounds (SVOCs) remaining on the back end of the chromatogram.

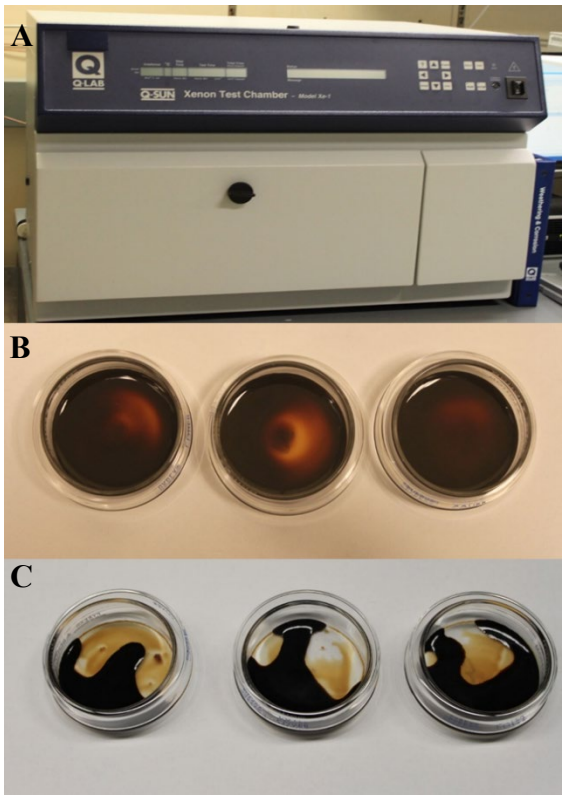

Figure 10. Q-Sun weathering chamber used for weathering experiments (A), HOOPS crude oil prior to weathering (B), and HOOPS after 12 hours of irradiation (C).

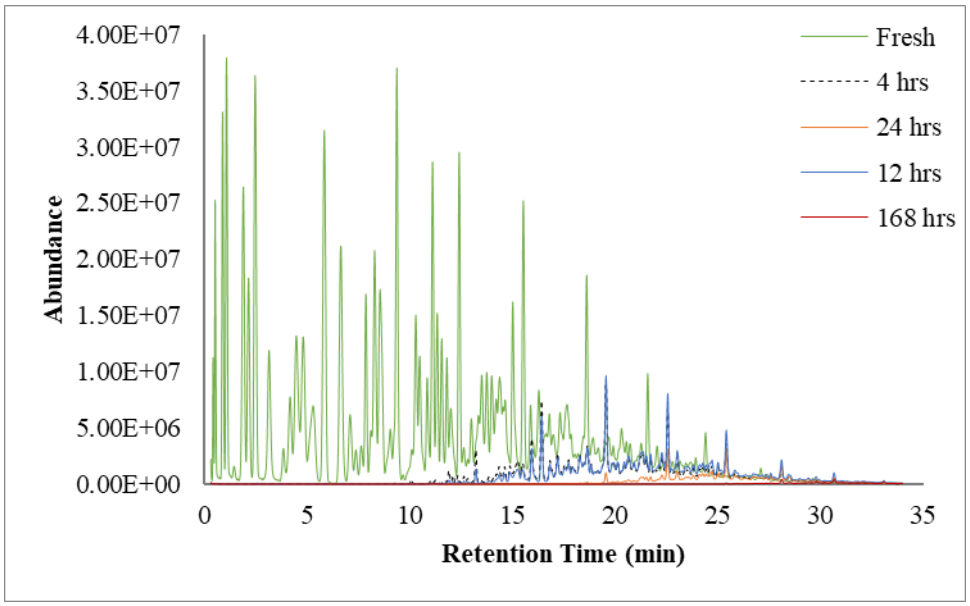

Figure 11. Overlaid chromatograms of fresh and weathered HOOPS crude oil for comparison of extracted headspaces.

Further headspace analysis revealed significant differences in the weathered samples compared to the fresh. Further headspace analysis (Fig. 12) revealed that after weathering for only 4 hours, the presence of polycyclic aromatic compounds appeared and increased with each irradiation time.

After 1 week of weathering (168 hrs), the headspace was predominately composed of alkanes (62%), whereas fresh HOOPS was mostly composed of branched alkanes (33%) and alkanes (28%). From this, it can be determined that as weathering of crude oil increases, the odor profile significantly changes, starting in as little as 4 hours. It was also found that as weathering time increased, the reproducibility of sample headspace decreased. Relative standard deviation (RSD) for triplicates of weathered for 168 hrs at peak 22.5 min (dodecane) was 173%, while the RSD for this same peak of fresh was 16%.

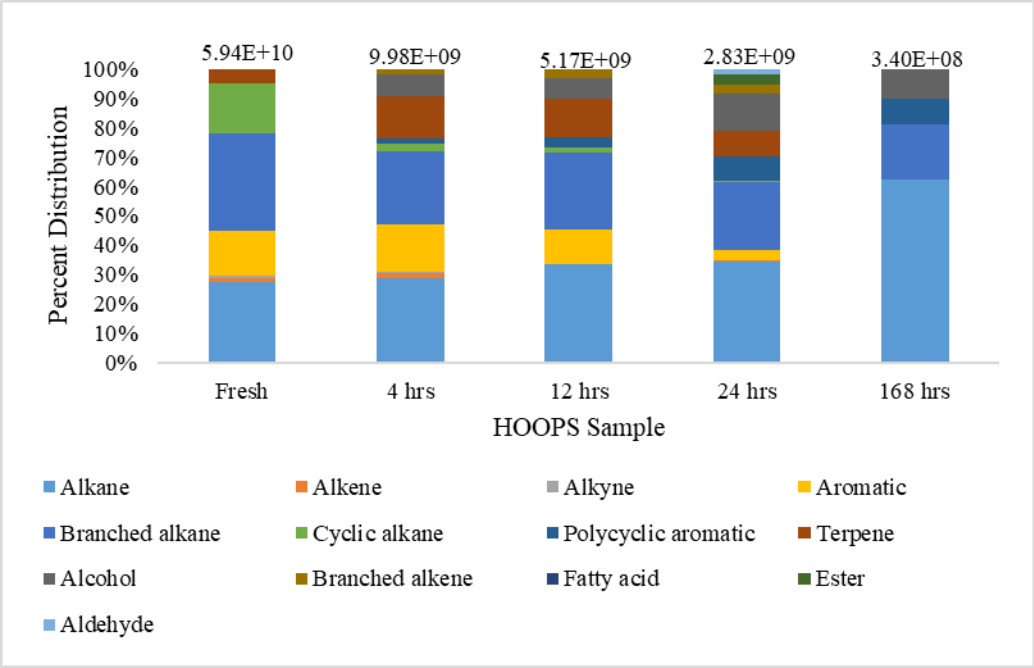

Figure 12. Compound class distribution of VOCs extracted from the headspace of simulated weathered HOOPS crude oil in comparison to fresh HOOPS crude oil. Values above bars represent total abundance in area counts.

2.1.2.2 Analysis of Weathered and Fresh Crude Oil using Chemometrics

The weathered series of HOOPS samples were aligned and matched by retention time. Peaks appearing in all three of the triplicates (above 1,000 peak height threshold) for a given weathering condition were kept in the first filtering step. Next, peaks that were not present in more than one time period were removed from the list; 77 peaks were identified after these criteria were applied. 37 peaks of these peaks were present at all time points, 18 compounds dropped out with increased sample weathering, 20 compounds were not detected in the fresh HOOPS sample, but were detected after weathering, and 2 peaks did not fall into any of these categories. The location of these compounds in the chromatogram are graphically displayed in Figure 13. As can be seen, the majority of the compounds that were lost were early-eluting HVOCs and those that remained detectable were the heavier, later-eluting SVOCs. The new compounds were spread throughout the chromatogram and are thought to be due to the breakdown of larger non-volatile molecules not detectable in the headspace of the crude oil.

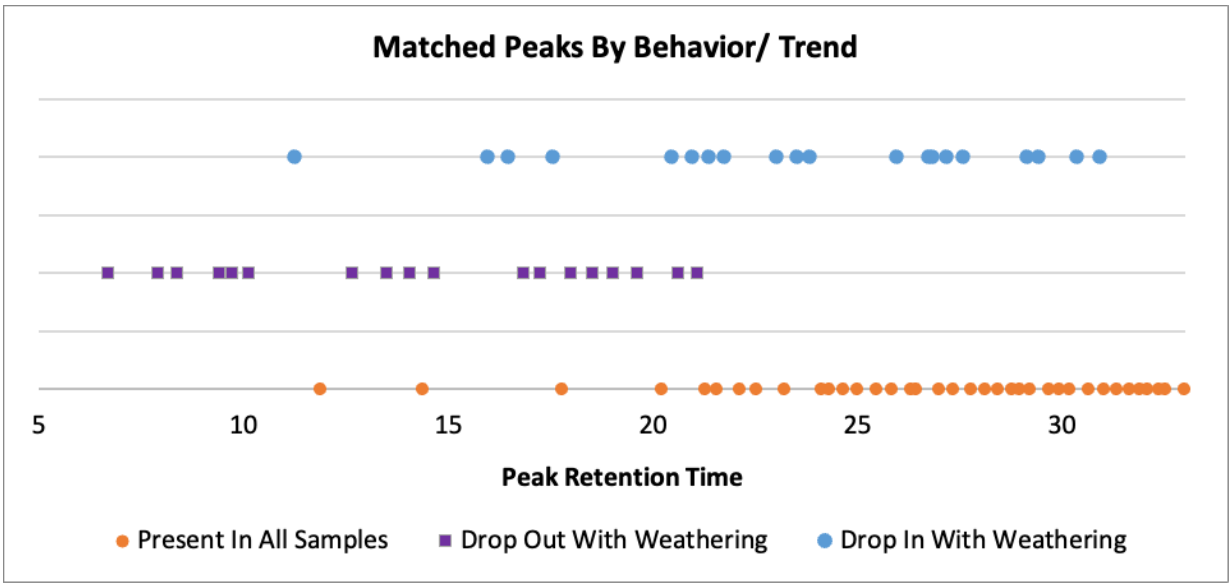

Figure 13. Retention time of compounds detected in fresh and weathered HOOPS crude oil, comparing those compounds present in all samples, those that were no longer detectable due to weathering, and those that appeared with increased weathering.

Of the 37 peaks found to be present within all of the weathering series samples, 18 peaks were found not to have statistically significantly different mean peak areas between sequential sampling periods. In this scenario, a peak with a 2 tailed t-test p-value > 0.05 between sequential sampling periods (0-4 hours, 4-12 hours, 12-168 hours) was determined to have a constant mean peak area.

- 18 peaks were not statistically significantly different between sequential sampling periods
- 9 peaks decreased in mean peak area between one or more sequential sampling periods
- 10 peaks did not exhibit a consistent trend in terms of peak area abundance

When comparing weathered HOOPS to both the fresh HOOPS and ANS, there were 15 compounds that were found to be present in the ANS & HOOPS samples. Of these 15 peaks, 14 peaks were present at a mean peak area value greater than 0, and 7 peaks were present in all of the triplicates of HOOPS samples at each weathering condition. These peaks were also found to display no statistically significant difference in abundance between fresh and weathered samples. Ultimately, these compounds may be volatile markers for crude oil that will remain present and detectable in the headspace. Future work will continue headspace analysis of a greater number of samples, both weathered and fresh, to determine the VOC markers in common between different crude oils types and conditions that could be the basis for canine generalization across many crude oil samples. Additionally, we will establish what compounds make the oils unique, allowing canines to be trained to discriminate between specific crude oils.

2.1.3 Task 1D – Headspace Analysis of Real Tarballs

Tarballs from the Delaware Bay oil spill in 2020 were collected on-site, and additionally NRL received several tarballs from different locations (Seacliff, CA; Ventura, CA; and Grand Isle, LA). Using the developed SPME-GC-MS method, the headspace of several tarballs were extracted and examined. Using this standard method, there was essentially nothing found in the headspace

(Figure 14). Extraction temperature was increased to 50 °C and with an increased extraction time of 18 hours, this did not result in the detection of additional compounds. Based on the simulated weathering of HOOPS, it is clear that many of VOCs in the headspace are lost after 24 hours, as compared to the fresh crude oil. However, due to canines' strong olfactory senses, they have previously been shown to be capable of detecting the minute amount of VOCs coming from weathered oil and tarballs. In future research, the headspace of simulated tarballs will be further investigated using addition sample preparation methods including dissolution in solvent.

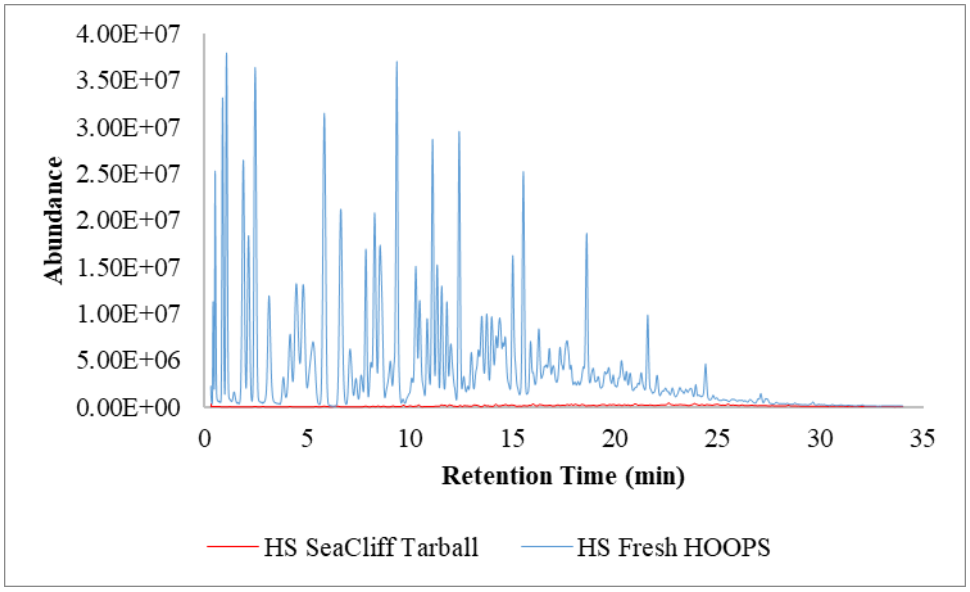

Figure 14. Overlaid chromatogram of Seacliff tarballs and fresh HOOPS crude oil extracted headspaces.

2.2 Phase 2 – Determination of Active Odorants for Canine Detection of Crude Oil

2.2.1 Task 2A – Fresh crude oil fractions to canines

2.2.1.1 Optimization of Collection Medium

In order to determine the optimal medium for collecting fractions of crude oil for canine trials, three sorbent materials were examined: Dukal gauze pad, Band-Aid gauze pad, and GetXent tube[9]. All sorbent materials were exposed to fresh HOOPS crude oil for 1.5 minutes using the experimental setup shown in Figure 15. The experimental setup was designed to mimic how the vapors from the headspace of crude oil would be collected using the fraction collector, with the exception of heat and fractionation. With an actual fraction collector, the sample would be heated, separated, and collected in set fractions. However, with this experimental design it was not feasible to separate or heat the sample. In this experiment, a 20 mL vial containing 5 mL of fresh HOOPS crude oil was placed inside of a Teflon container that was connected to a second Teflon container, which contained a second 20 mL vial containing one of the sorbent materials to collect the volatiles from the HOOPS crude oil. Nitrogen was used to purge the crude oil using a mass flow controller and a flow meter was used to monitor the flow rate, which was maintained at 5.3 mL/min (Dukal

[9] GetXent Tubes are composed of a patented blend of polymers capable of absorbing odor molecules and releasing that odor for up to six months. Website: https://getxent.com/product

and GetXent) and 5.4 mL/min (Band-Aid). The headspace of each sorbent material was extracted at 35 °C for 2 hrs on the same day as exposure (Day 0) to determine if any of the crude oil vapors were collected and again on Day 14 to determine if the sorbent materials were capable of retaining the headspace.

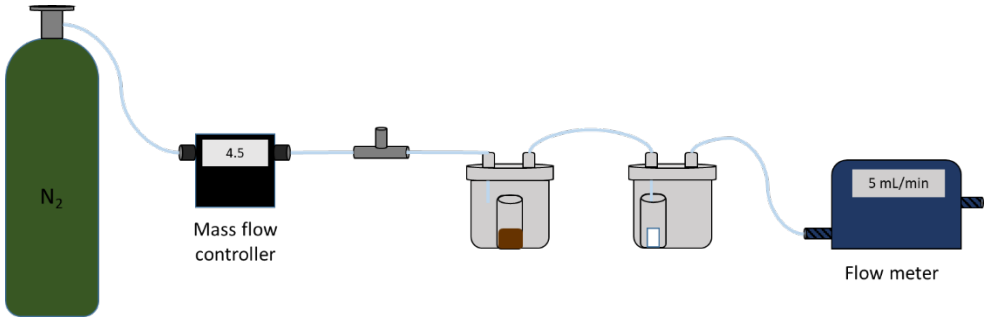

Figure 15. Schematic of experimental setup used for collection medium optimization.

Of the three materials, the greatest amount of odor was extracted from the GetXent tubes; however, there was a significant amount of loss from this and the other materials over the 14-day period (Fig. 16). Additionally, some cross-contamination between the samples and other chemicals stored in proximity was noted. Thus, these data imply that the storage system used for the 14 days was insufficient, allowing for odor loss and cross contamination from other chemicals stored in the same area. To improve the integrity of the samples over time, training aids were stored in a VOA vial, heat sealed in a mylar bag, and refrigerated. Ultimately, GetXent tubes were selected as the collection medium. All of the GetXent training materials were prepared, sealed in the VOA vials and mylar bags, and then shipped on dry ice to the canine trainer for testing.

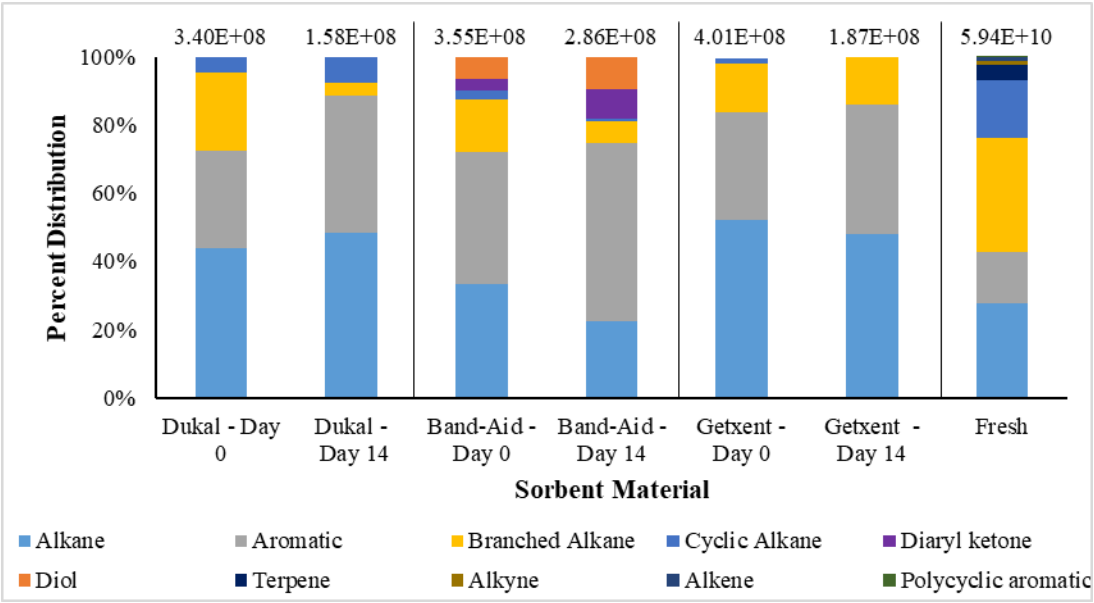

Figure 16. Compound class distribution of VOCs extracted from sorbent materials on Day 0 and Day 14 as compared to fresh HOOPS. Values above bars represent total abundace in area counts.

2.2.1.2 Odor Profile Collection

In order to determine which compounds canines use for detection of crude oil, canine testing probes were prepared from sections, or fractions, of the crude oil chromatograms. To collect these fractions, a fractionation technique was used to separate portions of the odor profile as they exit the GC. These fractions are then collected onto a medium which will be presented to canines for testing. A diagram showing the approach is given in Figure 17, where the headspace is extracted by SPME and placed in the heated inlet of the GC as usual. The VOC mixture is separated on the column by boiling point and then exits not into the MS, but instead on to a sorbent material placed at the end of the column in a vial. For testing, the full chromatogram from fresh HOOPS crude oil was collected onto GetXent tubes using the method pictured below.

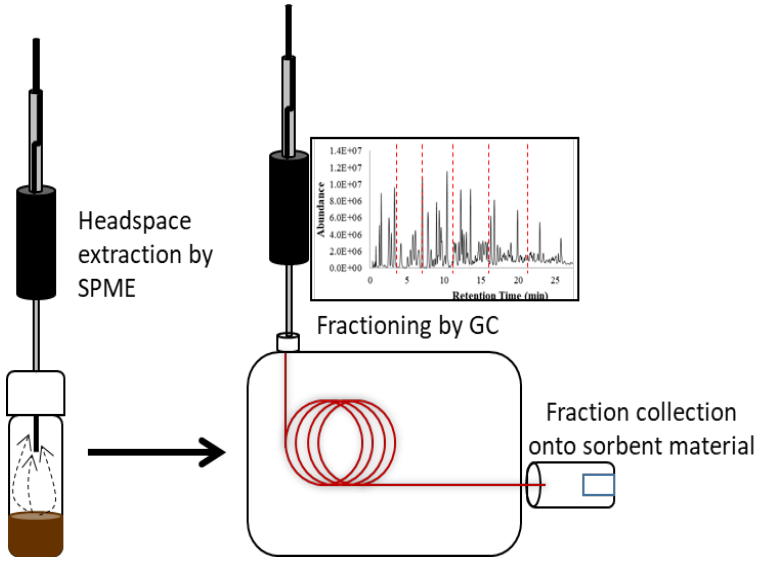

Figure 17. Schematic of experimental setup for fractionation and collection of odor signature.

2.2.1.3 Canine Testing: Fresh HOOPS Oil Fractions

Prior to testing the fractions of crude oil, several positive controls consisting of the full HOOPS chromatogram collected onto the GetXent tubes were sent to the trainer. Positive controls of HOOPS included one single collection of HOOPS, one double collection (two GC runs) of HOOPS, and one 5-hour soak made directly from the headspace of HOOPS (not using the GC). In addition, blank materials and negative controls prepared from blank GC runs were sent. Duplicates of each positive and negative control were provided for testing.

All controls, as well as blanks, were sent to Chiron K9 for on-site testing. The canines were tested utilizing stainless steel odor stands (Figure 18) offering 6 potential locations of target odor. Each stand held a sample within its VOA vial. All testing materials were assigned random numbers correlated to position on the stands allowing the canine testing to be double-blind, meaning neither the canine handler nor the test assessor knew the correct identity of the testing materials. The assistant observed through mirrored glass from the control room. Two canines were used for testing and have previously been trained to detect crude oil of differing origins and condition (fresh

and weathered). The canines utilized have had significant operational success, as discussed in Section 1 of this report.

Figure 18. Odor stands at Chiron K9.

Results from this preliminary testing session are given in Table 5. Two canines previously trained to detect crude oil were tested. Each canine was run by each sample in three separate testing sessions, equating to 12 chances for detection. Both canines alerted to all positive controls, with the exception of one canine, which missed one of the HOOPS GC x 1. There were no false alerts to the blank materials or to additional distractor odors that were included in the stands and were selected by the test provider (separate from the handler and assessor). Due to the one miss and because the fractions will have less total odor than the total chromatogram, it was determined that fractions needed to be made with double collections of crude oil headspace.

Table 5. Canine response to positive and negative controls of HOOPS oil on GetXent tubes.

Sample on GetXent tube	*Canine response (out of 12)*
HOOPS GC x1	11
HOOPS GC x2	12
HOOPS headspace (no GC)	12
Blank tubes (from GC)	0
Blank tubes (no GC)	0

To determine the portion of the chromatogram containing the key odorants for canine detection of fresh HOOPS oil, NRL prepared training aids from fractions of the oil headspace as described above. Three sets of fresh HOOPS training aids were prepared and tested by canines on-site at Chiron K9 on separate occasions. Additionally, each trial consisted of six distractors, six blanks, and five targets, as listed in Table 6. All distractors were prepared in the same manner as the

positive controls, where the headspace of the distractor materials was collected using SPME and then deposited through the GC onto the GetXent tube. The fractions were divided based on volatility (HVOC – 0-7.4 min, VOC – 7.4-12.6 min, and SVOC – 12.6-30 min) and two positive controls.

Table 6. Contents of fresh HOOPS training aids sent to Chiron K9.

Sample on GetXent tube	*Type*
Nitrile glove	Distractor
Glade wax melt	Distractor
Milkbone dog treat	Distractor
Rubber bands	Distractor
Peanut butter	Distractor
Oxiclean	Distractor
SPME-GC blank (2)	Blank
GC blank	Blank
Blank tubes (3)	Blank
HOOPS fraction 1	Target
HOOPS fraction 2	Target
HOOPS fraction 3	Target
HOOPS positive control (full GC run) (2)	Target

Results from the canine testing are given in Table 7 with associated chromatograms in Figure 19. No false alerts to distractors or blanks were noted. Each canine alerted to each fraction in at least two of the three trials, indicating that they are capable of using parts of the odor signature for detection. This is likely because the canines have been taught to generalize across many types and conditions of crude oil. The canines alerted to Fraction 3 in all trials. This fraction is the fraction that remains the most consistent during weathering, and is thus apparently the most recognizable portion of the odor profile. Future testing will be done with canines trained to only fresh crude oil or only to a single crude oil type.

Table 7. Canine responses to fresh HOOPS training aids. "A" denotes a positive alert, while "N" denotes no alert.

	Fraction 1		Fraction 2		Fraction 3	
	Canine 1	Canine 2	Canine 1	Canine 2	Canine 1	Canine 2
Fresh Trial 1	A	A	N	N	A	A
Fresh Trial 2	A	A	A	A	A	A
Fresh Trial 3	N	N	A	A	A	A
Total Alerts	4		4		6	
Alert Response %	67%		67%		100%	

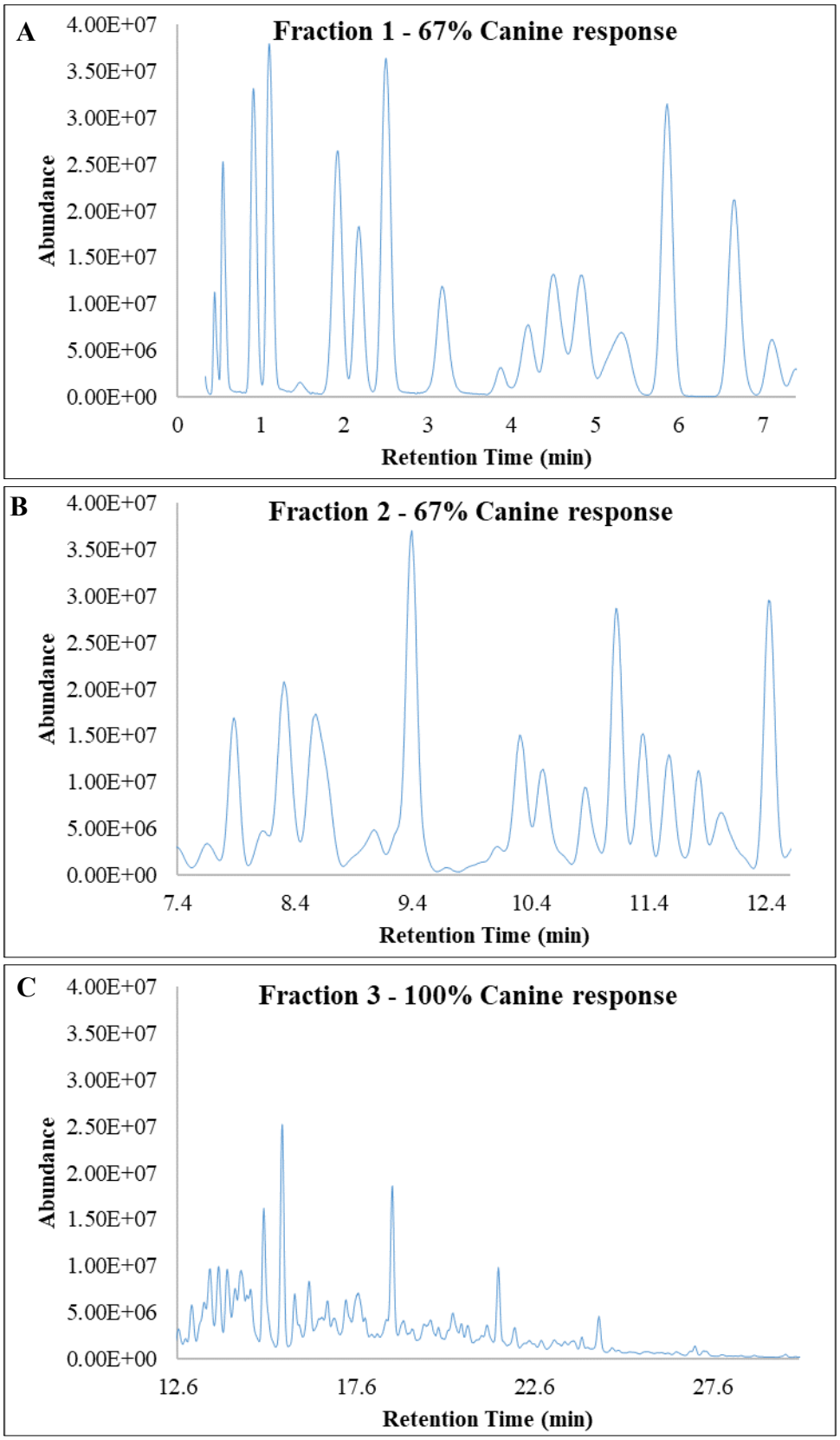

Figure 19. Chromatograms of fresh HOOPS fractions and their respective canine responses. Fraction 1: 0 – 7.4 min (A), fraction 2: 7.4 – 12.6 min (B), and fraction 3: 12.6 – 30 min (C).

2.2.2 Task 2B – Weathered HOOPS Oil Fractions to Canines

Testing of the weathered HOOPS odor profile was carried out in the same manner as the fresh trials. As such, three trials of weathered HOOPS training aids were tested by canines on-site at Chiron K9. Each training aid trial consisted of six distractors, six blanks, and five targets as described in Table 8. Similar to the fresh training aids, each set of samples were mailed overnight on dry ice. HOOPS was weathered for 12 hours and targets consisted of three fractions of weathered HOOPS with collection times based on fresh fractions.

Table 8. Contents of weathered HOOPS training aids sent to Chiron K9.

Sample on GetXent tube	*Type*
Nitrile glove	Distractor
Glade wax melt	Distractor
Milkbone dog treat	Distractor
Rubber bands	Distractor
Peanut butter	Distractor
Oxiclean	Distractor
SPME-GC blank (2)	Blank
GC blank	Blank
Blank tubes (3)	Blank
Weathered HOOPS fraction 1	Target
Weathered HOOPS fraction 2	Target
Weathered HOOPS fraction 3	Target
Fresh HOOPS positive control (2)	Target

Results from the canine trials are given below in Table 9 with associated chromatograms in Figure 20. Canines alerted 100% to Fraction 1, 67% to Fraction 2, and 67% to Fraction 3 (Table 9). Both canines alerted to distractors made from rubber bands, but no other false alerts to distractors or blanks were noted. The fact that canines readily alerted to Fraction 1 was very interesting, as very little was detected in the headspace of Fraction 1 from the 12 hour-weathered sample. These canines have also been trained to detect tarballs and other highly weathered oil projects. Fraction 1 is very similar to the tarballs and this previous training is likely responsible for its detection. Fractions 2 and 3 were detectable, but more challenging at times. Changes to the odor profile due to the addition of new compounds occurs in Fractions 2 and 3, possibly making detection more difficult. More information is needed to better understand canine detection of weathered oil, which could be gathered through the testing of canines trained solely in the detection of fresh, unweathered oil. Additional testing with the odor profile of tarballs may also shed more light on these results.

Table 9. Canine responses to HOOPS training aids weathered for 12 hrs. "A" represents positive alerts, while "N" denotes no alert.

	Fraction 1		Fraction 2		Fraction 3	
	Canine 1	Canine 2	Canine 1	Canine 2	Canine 1	Canine 2
Weathered Trial 1	A	A	N	N	A	A
Weathered Trial 2	A	A	A	A	A	A
Weathered Trial 3	A	A	A	A	N	N
Total Alerts	6		4		4	
Alert Response %	100%		67%		67%	

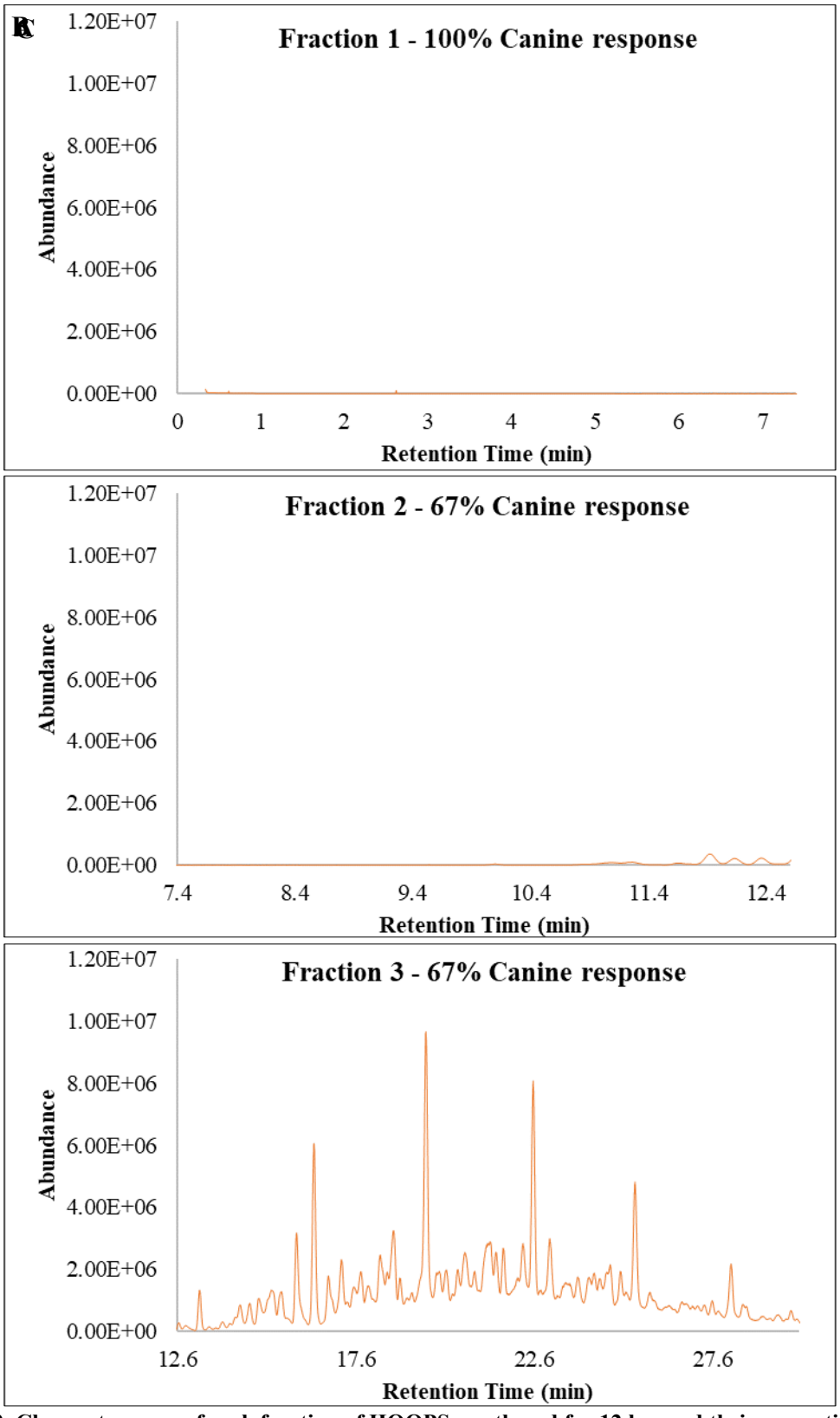

Figure 20. Chromatograms of each fraction of HOOPS weathered for 12 hrs and their respective canine responses to training aids. Fraction 1: 0 – 7.4 min (A), fraction 2: 7.4 – 12.6 min (B), and fraction 3: 12.6 – 30 min (C).

2.3 Phase 4 – Canine Training Protocols for Water-Sequestered Oil

Chiron K9 developed a novel underwater training device which utilizes crude oil releasing the volatile components and pushing them out through a pipe placed within a water source. Volatiles are then detected at the surface of the water source.

Initial canine training and testing in the laboratory utilized buckets of water containing Training Aid Delivery Device (TADDs)[10] in a 3-choice lineup. One TADD contained the oil and the remaining two were controls (no oil). Each TADD was placed in a 2-gallon bucket with water and canines worked the lineup successfully.

Once the canines were able to detect and indicate the presence of oil under water within a TADD in a bucket of water the training progressed to bubbled air in buckets. An air compressor was connected to three stainless steel containers. Each container was connected to a 5-gallon bucket by tubing so that positive air was blown through the system and bubbled underwater in the buckets. One of the containers had oil contained in a Mason jar and two containers were blank (Fig. 21).

Figure 21. 5-gallon bubbler device.

Following laboratory testing, an underwater detection device was developed for field-based testing. The device is composed of an air compressor, air tight container for oil, and a 100 ft hose (Fig. 22). The system was set up with the air/odor supply on the shoreline. The outlet pipe was deployed into the lake and initially the end of the pipe was weighted to deliver the odor into the water column just below the lake surface (Fig. 23). After several trials the system was reconfigured to anchor the entire pipe to the lake bed (Fig. 24). The rate of air supply was controlled on land

[10] Maughan, Michele Nancy. "Methods of using training aid delivery devices (tadd)." U.S. Patent No. 10,813,342. 27 Oct. 2020.

and varied during the tests with one set of tests that had low, intermittent supply rates. In these shallow water depths (<5 feet) this did not affect successful detection by the canine.

Figure 22. Underwater detection training device

Figure 23. Initial set up with a floating pipe and subsurface mid-level water column delivery.

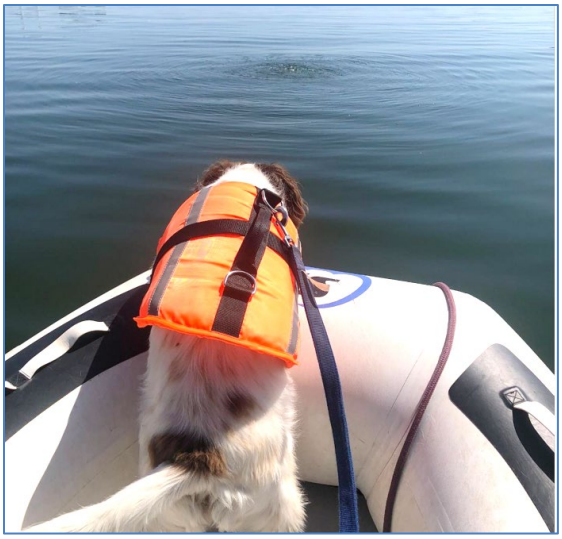

Figure 24. On-water subsurface oil odor detection training with the pipe anchored to the lake bed. Odor bubbles can be seen surfacing directly ahead of the boat.

After the field testing of the system was completed, a canine was trained to work from the bow of a small electric-powered boat to search for the odor source (Fig. 23). Preliminary protocols for training canines in this technique were evaluated by a systematic approach adjusted to provide an effective and efficient set of training steps for this detection application. The boat was positioned downwind of the submerged source, which had no visual markers, and searches were conducted with both zig zag and linear patterns (Fig. 25). The handler/boat operator observed the changes in behavior of the canine to steer towards the source. In the final phase of the training the canine was encouraged to communicate to the boat operator the direction to the source. This was a significant innovation in communication between the canine and the handler.

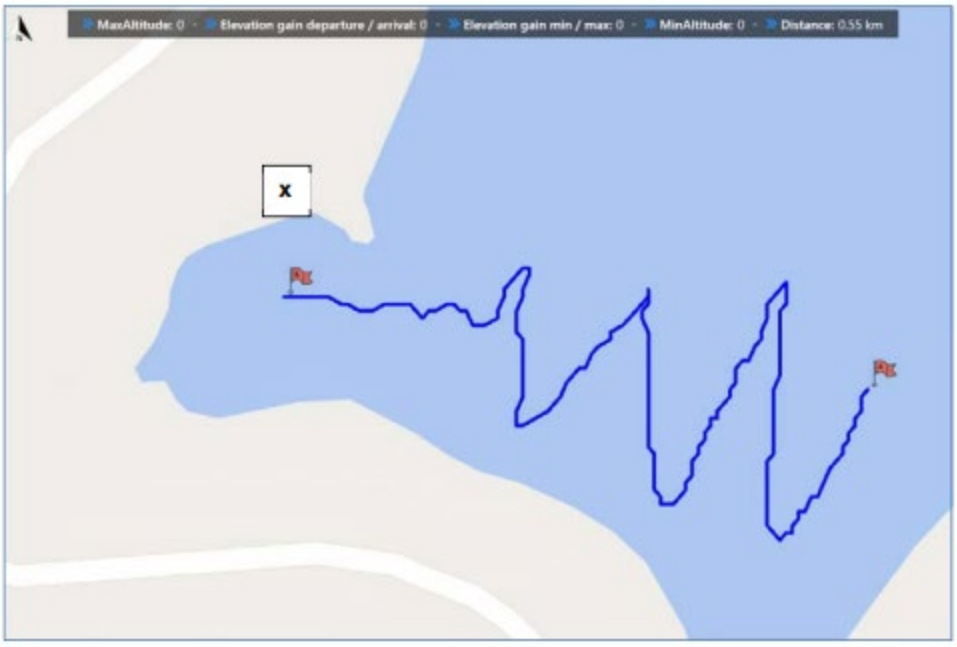

Figure 25. Example of zig-zag search pattern shown by GPS track line. The location of the shore-side delivery system is marked by the X.

3 CONCLUSIONS & FUTURE WORK

In Year 1 (Y1), a SPME-GC-MS method was developed and optimized using fresh HOOPS crude oil, allowing for the characterization of the odor profiles of HOOPS and ANS crude oils. A comparison of the odor profiles of the two oils showed many compounds in common, though there were enough differences to correctly categorize samples as HOOPS or ANS using individual peak comparison by Spearman Rank Correlation or using the whole chromatogram by 3-D Covariance. Changes in the odor profile due to photodegrative weathering was also assessed. Results showed a notable loss of the early-eluting HVOCs in addition to the appearance of several poly-cyclic compounds.

In Phase 2, canine testing was carried out to determine which portion of the odor profile was used in oil detection. It was shown that the canines are capable of distinguishing crude oil from any fraction of the odor profile. This capability allows the canines to readily generalize between oils of different types and conditions. However, the canines most readily detected the heavier SVOC fraction of the fresh oil, likely because this fraction was the most consistent between types of oil and degree of weathering. Additionally, the canines readily detected the HVOC section of the weathered section. The authors hypothesize that this section is similar in odor to tarballs, to which the canines had previously been trained.

In the final portion of the research, an underwater training device was developed, allowing for canines to be trained to locate submerged oil in a body of water from the shoreline or boat, without risk of contaminating the water with actual oil. Prototype and on-shore testing of this device was

completed in Year 1 and a patent disclosure will be filed. Future research will include device usage from a watercraft.

Due to COVID-19 and major delays caused by NRL administration processes, all tasks for Y1 were not completed. A planned direction for a Year 2, would be to complete headspace analysis of degraded or submerged crude oils, which would include weathering of ANS, headspace analysis of tarballs, biodegradation of crude oil, submerged crude oil, and create an archive of crude oil odor profiles. This would then allow for more probing of canine detection capabilities.

A logical follow on for the training of canines to detect sunken and submerged oils using the device developed in Y1 would be to conduct field trials with different oil types, at different depths, and in still and moving water bodies. Field trials would be designed based on calculated parameters and models for (1) rise times for the gas, (2) the radii of the surfacing plume areas, and (3) current transport drift distances and dye tracers would be used to verify the observed surface expressions of the plumes at the time of the trials.