ENVIRONMENTALLY BENIGN MITIGATION OF MICROBIOLOGICALLY INFLUENCED CORROSION (MIC)

FIFTH QUARTER REPORT

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ABSTRACT

Title: Environmentally Benign Mitigation of Microbiologically Influenced Corrosion (MIC)

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Objective: The overall program objective is to develop and evaluate environmentally benign agents or products that are effective in the prevention, inhibition, and mitigation of microbially influenced corrosion (MIC) in the internal surfaces of metallic natural gas pipelines. The goal is to develop one or more environmentally benign (a.k.a. "green") products that can be applied to maintain the structure and dependability of the natural gas infrastructure.

Approach: The technical approach for this quarter includes the application of new methods of *Capsicum sp*. (pepper) extraction by soxhlet method and analysis of a new set of extracts by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC); isolation and cultivation of MIC-causing microorganisms from corroded pipeline samples; and evaluation of antimicrobial activities of the old set of pepper extracts in comparison with major components of known biocides and corrosion inhibitors.

Results: Twelve new extracts from three varieties of *Capsicum sp*. (Serrano, Habanero, and Chile de Arbol) were obtained by soxhlet extraction using 4 different solvents. Results of TLC done on these extracts showed the presence of capsaicin and some phenolic compounds, while that of HPLC detected capsaicin and dihydrocapsaicin peaks. More tests will be done to determine specific components. Additional isolates from the group of heterotrophic, acid-producing, denitrifying and sulfate-reducing bacteria were obtained from the pipeline samples submitted by gas companies. Isolates of interest will be used in subsequent antimicrobial testing and test-loop simulation system experiments. Results of antimicrobial screening of *Capsicum sp*. extracts and components of known commercial biocides showed comparable activities when tested against two strains of sulfate-reducing bacteria.

Conclusions: The testing for the isolation of a novel biocide or biofilm inhibitors is progressing on schedule. The isolated extracts are showing activities against the sulfate-reducing bacteria and the tests are progressing with the other microorganisms. The fractionation of the extracts using the HPLC is progressing rapidly.

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INTRODUCTION

The overall objective of this project is to develop, test, and apply environmentally benign agent(s) to control microbial-caused corrosion on the internal surfaces of metal (iron or steel) pipes used for natural gas transmission. The overall hypothesis is that agents exist in nature that inhibit some or all of the steps executed by microorganisms in the formation of biofilm. As biofilm formation is an absolute prerequisite for the initiation and production of microbially influenced corrosion (MIC), blocking biofilm formation or propagation will block or mitigate MIC.

The general approach is to evaluate natural products isolated from plants, and, possibly, animals or microorganisms, for their abilities to block the attachment, physiology, or reproduction of microbial agents that are responsible for MIC. The first natural product to be tested is the oil that can be extracted from the seeds and pods of pepper plants. These plants are members of the Genus *Capsicum.* The effective components or constituents of this product will then be tested for its environmental impact and effects, effective concentrations, modes of application, and stability against isolated MIC microorganisms under simulated field conditions. A commercially viable agent that aids in MIC control and is environmentally friendly is the ultimate target, with preliminary data to determine commercialization potential and cost benefits.

EXECUTIVE SUMMARY

The main goal of this project is to develop an environmentally benign compound that could prevent and/or control microbially influenced corrosion (MIC) in the interior of metal gas pipelines.

The majority of this quarter's activities were directed towards Objectives No. 1) to isolate and cultivate MIC-causing microorganisms / biofilm, and 4) to evaluate pepper oil components to inhibit and mitigate biofilm formation and MIC.

MIC samples from corroded gas production and transmission pipelines and storage tanks submitted by gas companies were used to isolate bacteria from the groups of acid producers, heterotrophs, denitrifiers, sulfate reducers, iron reducers and methanogens. Eleven prospective isolates were processed for identification using the 16S rRNA sequencing, showing that they belong to the Genera *Ralstonia, Shewanella, Desulfovibrio, Pseudomonas, Bacillus* and *Aeromonas*. More isolation and culture work is being done on the new corrosion samples from the field.

Antimicrobial screening using extracts and components of known commercial biocides and corrosion inhibitors showed comparable activities of the H1M, S1E and S1M *Capsicum* extracts with glutaraldehyde that has a minimum inhibitory concentration of 156 μg/mL when tested against *Desulfovibrio vulgaris*. All of the four extracts demonstrated lower inhibitory concentration of 78 μg/mL than glutaraldehyde with 156 μ/mL against *D. desulfuricans.* Benzalkonium chloride and zinc chloride, however, showed stronger inhibitory activities against the two organisms with 19.5 and 1.17 μg/mL, respectively. Sodium molybdate and monochloramine with 2.5 – 5.0 mg/mL have lower antimicrobial activity than the extracts.

A total of 12 new extracts from three varieties of *Capsicum sp*. (such as, Serrano, Habanero and Chile de Arbol C) were obtained by soxhlet extraction using hexane, methylene chloride, aqueous acid, and aqueous alkali. Results of TLC showed the presence of capsaicin and phenolic compounds that still need to be identified. HPLC detected capsaicin and dihydrocapsaicin peaks. More analytical tests will be done to determine specific components of each extract.

EXPERIMENTAL METHODS

Objective 1 – Isolate and cultivate MIC-causing microorganisms / biofilm

Condensates and biofilm-containing samples from the corroded gas gathering lines, production lines, and/or storage fields were submitted by Laclede Gas Laboratory (St. Louis, MO), Tom Brown, Inc. (Meeker, CO), Williams Production (Parachute, CO), Colorado Interstate Gas (Richfield, KS), Gulf South Pipeline Co. (Carthage, TX). They were processed anaerobically using the Most Probable Number (MPN) method (Garthright 2001) and standard anaerobic techniques (Holdeman, 1977; Balch and Wolfe, 1976) to isolate microorganisms from the groups of acid-producing bacteria (APB), heterotrophic bacteria, denitrifying bacteria, sulfate-reducing bacteria (SRB), iron-reducing bacteria, and methanogenic bacteria. Salinity and pH of the six types of media that correspond to each bacterial group were adjusted according to that of the original sample. The tubes were incubated at 30^{o}C and MPN was determined after two and four weeks. 100 uL aliquot from the MPN tube was streaked onto corresponding solid media and incubated anaerobically at 30^{o}C. Isolates were purified by repeated streaking on agar plates until isolated colonies were obtained. Pure isolates were processed by Microbial Insights, Inc. (Rockford, TN) for identification using the 16S rRNA gene sequencing technique.

Objective 4 – Evaluate pepper oil components to inhibit and mitigate biofilm formation and MIC

The microdilution assay method (Eloff, 1998) was used to determine the minimum inhibitory concentration of the pepper components. The pepper extracts and pure compounds were serially diluted 50% with sterile distilled water in 96-well microtiter plates. For the inoculum, an overnight bacterial culture was centrifuged at 4,000 rpm for five minutes, washed with a 50mM potassium phosphate buffer, pH 7.0, and the optical density was adjusted at 600 nm to obtain a final cell concentration of 10^6 cfu/mL per well. ATCC # 1249 Modified Baar's Medium was used as a source of cell nutrients. The plates were then placed inside a modular anaerobic chamber, purged with nitrogen gas, and incubated at 30^{o}C for *D. vulgaris* and 37^{o}C for *D. desulfuricans*. Optical density at 600 nm was measured using the MRX II (Dynex Technologies) plate reader from 0 up to 168 hours. MIC was determined as the minimum concentration of the test compound limiting turbidity to <0.05 absorbance at

600nm (Cai et al, 2000). Minimum bactericidal concentration (MBC) was determined by taking a 100 μL sample from the wells where MIC was found, and the next higher concentration to it. Samples from experimental and untreated wells were serially diluted, plated out, and incubated in appropriate anaerobic conditions. Colonies were counted after 48-72 hours, and MBC was defined as the minimum concentration where 99% of the organisms were killed.

Three varieties of *Capsicum sp*. commonly known as Serrano, Habanero and Chile de Arbol were used to obtain new extracts. The soxhlet extraction method was used using hexane, methylene chloride, aqueous acid (pH 2.0), and aqueous alkali (pH 12.0) one at a time with each new set of samples. Fresh Serrano and Habanero peppers were ground in a spice grinder, dried at 70^{o}C overnight, and ground again to form a powder. Chile de Arbol that were purchased dried were powdered using a spice grinder. Each type of pepper was extracted with each solvent non-sequentially for a total of 12 separate extractions per pepper. Forty grams of pepper powder were extracted with 350 mL of solvent conducted in four replicates. The extraction process was completed in 18 hours and the replicates were combined together. The extracts were concentrated using a Buchii Rotavapor (Model R-205), and excess solvent was evaporated using TurboVap LV Evaporator.

The concentrated extracts were subjected to thin-layer chromatography using normal phase Whatman KC-18F Silica Gel 60A, 20 x 20 cm plates with a layer thickness of 200 μm. The plate was spotted with 5ul samples and standards. The mobile phase consisted of 70:10:17:3 mL of chloroform : ethanol : ethyl acetate : hexane. Fifteen milliliters of this mobile phase was allowed to equilibrate in the TLC chamber for 10 minutes. Then the solvent was allowed to travel into the plate until it reached ½ inch from the top. The plate was air-dried before being sprayed with the developing reagent. Anisaldehyde (5% in acid alcohol) was the first reagent that was sprayed onto the plate. Once dried, the plate was placed in a 100^{o}C oven for one minute. This reagent was used to detect phenols, essential oil components or terpenes, steroids, mycotoxins, antibodies, and many other compounds. The same plate was sprayed again with another reagent called phosphomolybdic acid to check for the presence of phenols, terpenes, and steroids. Samples (5 μL each) were also spotted on a reverse-phase TLC plate (Whatman KC18). The mobile phase consisted of 80:15:5 mL

acetonitrile : ethanol : water, and the developing reagent used was Dragendorff"s Reagent that determined the presence of lipids (Figure 4).

The extracts were then analyzed by HPLC equipped with a photodiode detector. A Supelcosil LC-18 (25cm x 4.6mm; five-micron particle size; Supelco) analytical column was used at 50^{o}C temperature. Peaks were obtained between 28 minutes and 51 minutes. These peaks will be fraction collected for further analysis. The eluent was composed of: Solvent A .01 M H3PO4 (80%), Solvent B Methanol (20%), at a flow rate of 1.0ml/min, holding for two minutes and ramping gradually by 51 minutes to obtain 0% solvent A and 100% solvent B and holding for four minutes. A wavelength of 285 nm was used to detect the Capsicum and dihydrocapsaicin peaks.

RESULTS AND DISCUSSION

Corrosion samples from gas production and transmission pipelines, and storage tanks submitted by gas companies were used to isolate bacteria from the groups of acid producers, heterotrophs, denitrifiers, sulfate reducers, iron reducers, and methanogens. Eleven prospective isolates were identified using the 16S rRNA gene-sequencing technique, and they were found to be in the Genera *Ralstonia, Shewanella, Desulfovibrio, Pseudomonas, Bacillus* and *Aeromonas*. These cultures are being maintained for future antimicrobial and test-loop simulation system studies. More isolation and culture work is being done on the new corrosion samples from the field.

Table 1 displays the results of antimicrobial screening using extracts and components of known commercial biocides and corrosion inhibitors, showing comparable activities of the H1M, S1E, and S1M *Capsicum* extracts with glutaraldehyde that has a minimum inhibitory concentration of 156 μg/mL when tested against *Desulfovibrio vulgaris.* All of the four extracts demonstrated lower inhibitory concentration of 78 μg/mL than glutaraldehyde with 156 μ/mL against *D. desulfuricans.* Benzalkonium chloride and zinc chloride, however, showed stronger inhibitory activities against the two organisms, with 19.5 and 1.17 μg/mL, respectively. Sodium molybdate and monochloramine with 2.5 – 5.0 mg/mL have lower antimicrobial activity than the extracts.

Table 1. Minimum Inhibitory Concentration (MIC)and Minimum Bactericidal Concentration (MBC) of Biocides and *Capsicum sp*. Compounds

Agents	*D. vulgaris*		*D. desulfuricans*	
	MIC	MBC	MIC	MBC
H1E	312.5	625.0	78.0	312.5
H1M	156.0	312.5	78.0	312.5
S1E	156.0	312.5	78.0	156.0
S1M	156.0	156.0	78.0	156.0
Capsaicin	156.0	312.5	312.5	1,250.0
Dihydrocapsaicin	240.0	480.0	240.0	960.0
Caffeic Acid	302.5	302.5	302.5	302.5
Cholorogenic Acid	312.5	312.5	312.5	625.0
Sitosterol	4.11	16.43	65.75	65.75
Monochloramine	2500.0	2500.0	5000.0	5000.0
Glutaraldehyde	156.0	156.0	156.0	312.5
Benzalkonium chloride	19.5	19.5	19.5	19.5
Zinc chloride	1.17	1.17	1.17	1.17
Sodium molybdate	2500.0	2500.0	2500.0	2500.0
Ampicillin	6.25	6.25	6.25	25.0
Kanamycin	>200.0	ND	>200.0	ND
Methanol	0.625%	1.25%	1.25%	2.5%

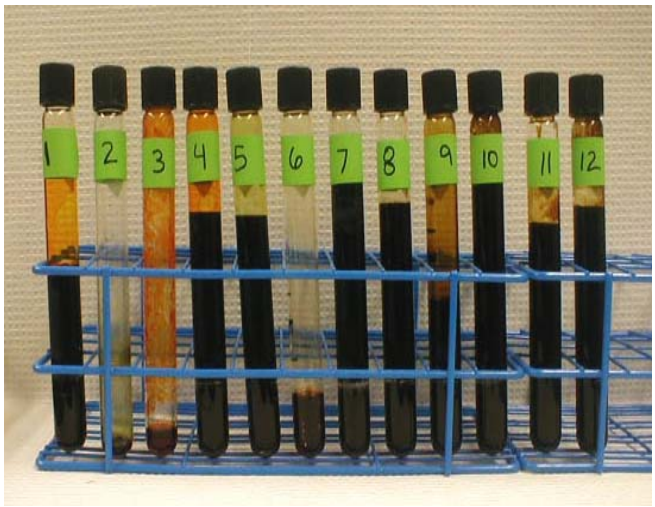
Note: ND – Not Determined
Concentrations are in μg/mL unless otherwise stated.

H1E, H1M, S1E, S1M – *Capsicum sp*. crude extracts

A total of 12 new extracts from three varieties of *Capsicum sp*. (such as, Serrano, Habanero and Chile de Arbol) were obtained by soxhlet extraction using hexane, methylene chloride, aqueous acid, and aqueous alkali. Figure 1 shows the photos of the peppers used while Figure 2 shows the extracts from them.

Chile de Arbol Habanero Serrano

Figure 1. *Capsicum sp*. varieties used in soxhlet extraction.



Soxhlet Extraction of Fresh/Dry *Capsicum sp*.

1. Chile de Arbol with Hexane
2. Serrano with Hexane
3. Habanero with Hexane
4. Chile de Arbol with Methylene Chloride
5. Serrano with Methylene Chloride
6. Habanero with Methylene Chloride
7. Chile de Arbol with Aqueous Acid
8. Serrano with Aqueous Acid
9. Habanero with Aqueous Acid
10. Chile de Arbol with Aqueous Alkali
11. Serrano with Aqueous Alkali
12. Habanero with Aqueous Alkali

Figure 2. Extracts obtained using different solvents.

TLC revealed the presence of capsaicin and phenolic compounds (Figure 3). More testing has to be done to check what other specific compounds are present in the samples and to double check spots that are questionable (Figure 4).

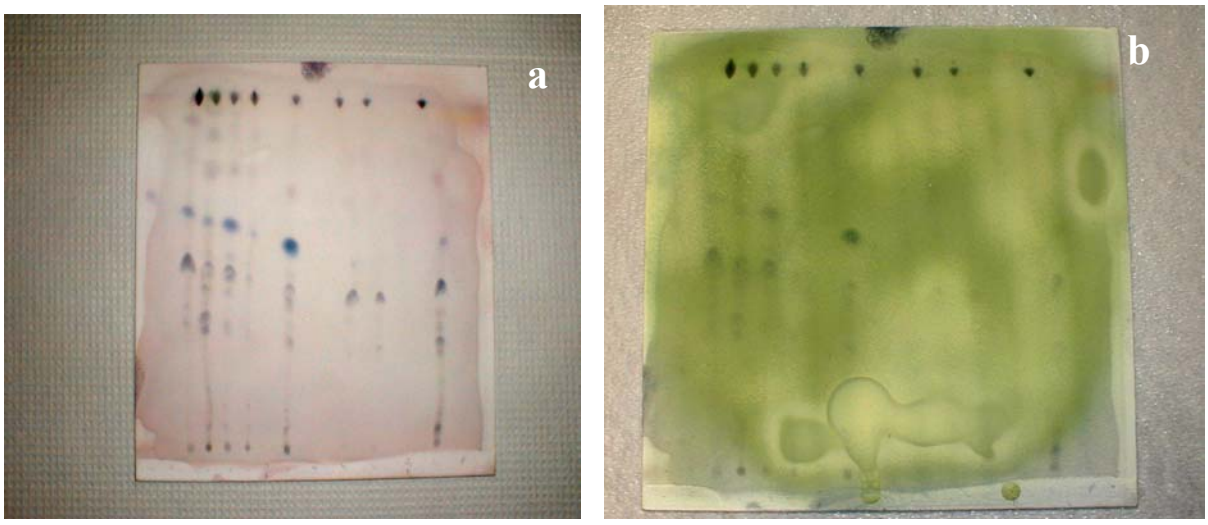


Figure 3. a) Sprayed with anisaldehyde, the bright blue color is the capsaicin compound. The others might be the phenols, steroids, or flavones. b) The same plate as one at left but sprayed with phosphomolybdic acid to detect phenols, terpenes, and steroids. It showed some spots on top and a few light ones throughout the plate. More testing with standards will have to be done to prove that these spots represent the compounds in question.

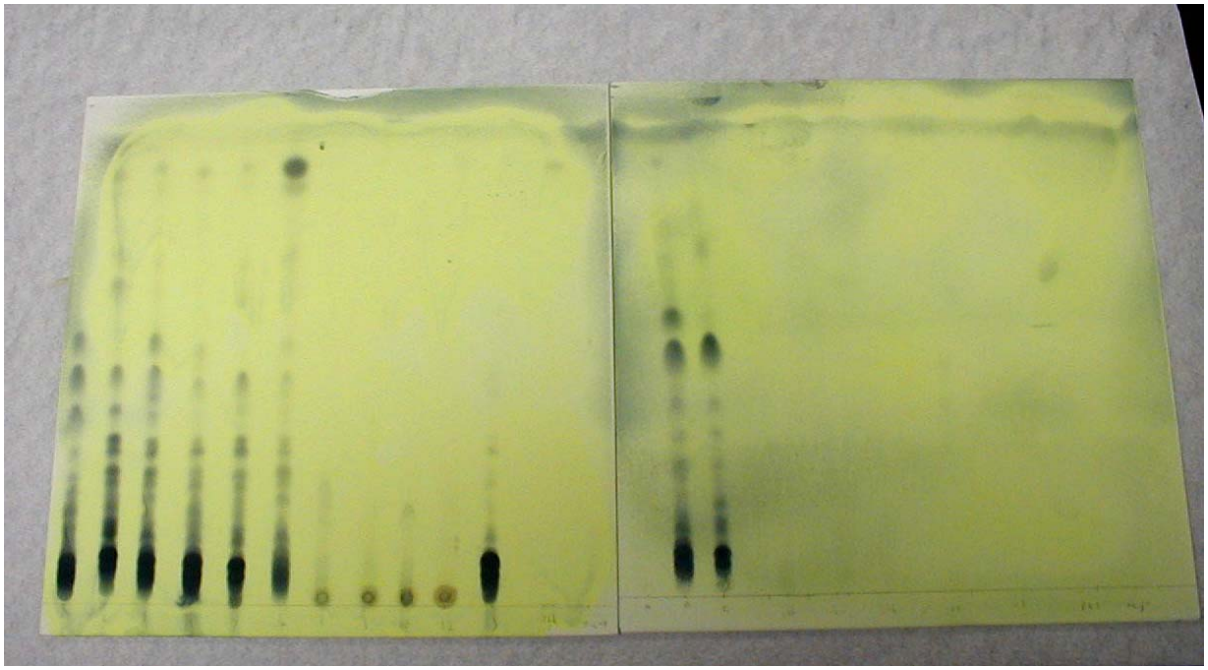


Figure 4. Reverse-phase TLC plates developed using Dragendorff's Reagent to determine the presence of lipids. The lines on the plates on the left are the samples; on the right are standards. The only standard that showed a clear spot was Oleic Acid. This experiment will have to be rerun with a lower concentration of the samples for better resolution. The peak in question is in the middle.

Results of HPLC analysis detected capsaicin and dihydrocapsaicin peaks in all of the samples tested.

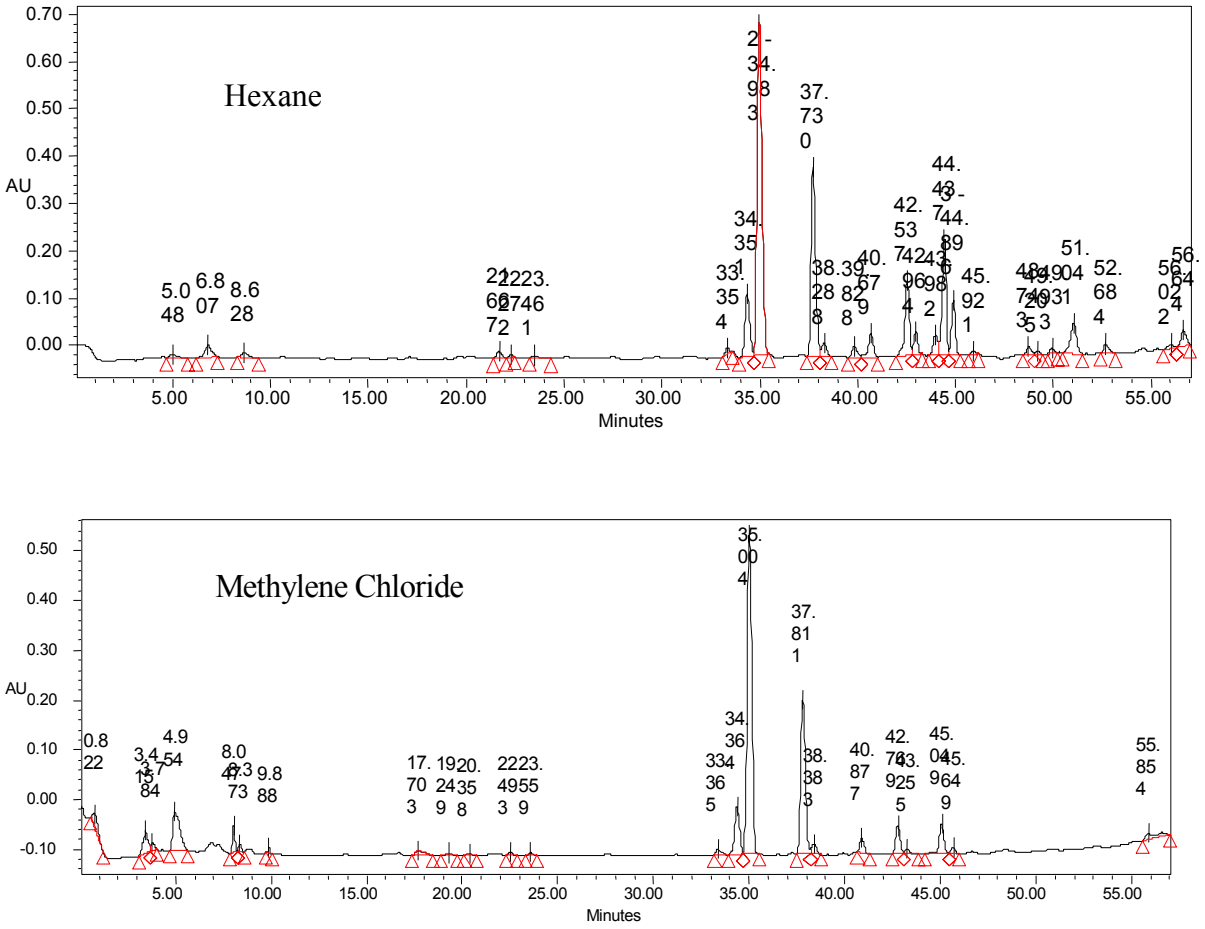


Figure 5. Chromatogram of Chile de Arbol extracts after HPLC analysis

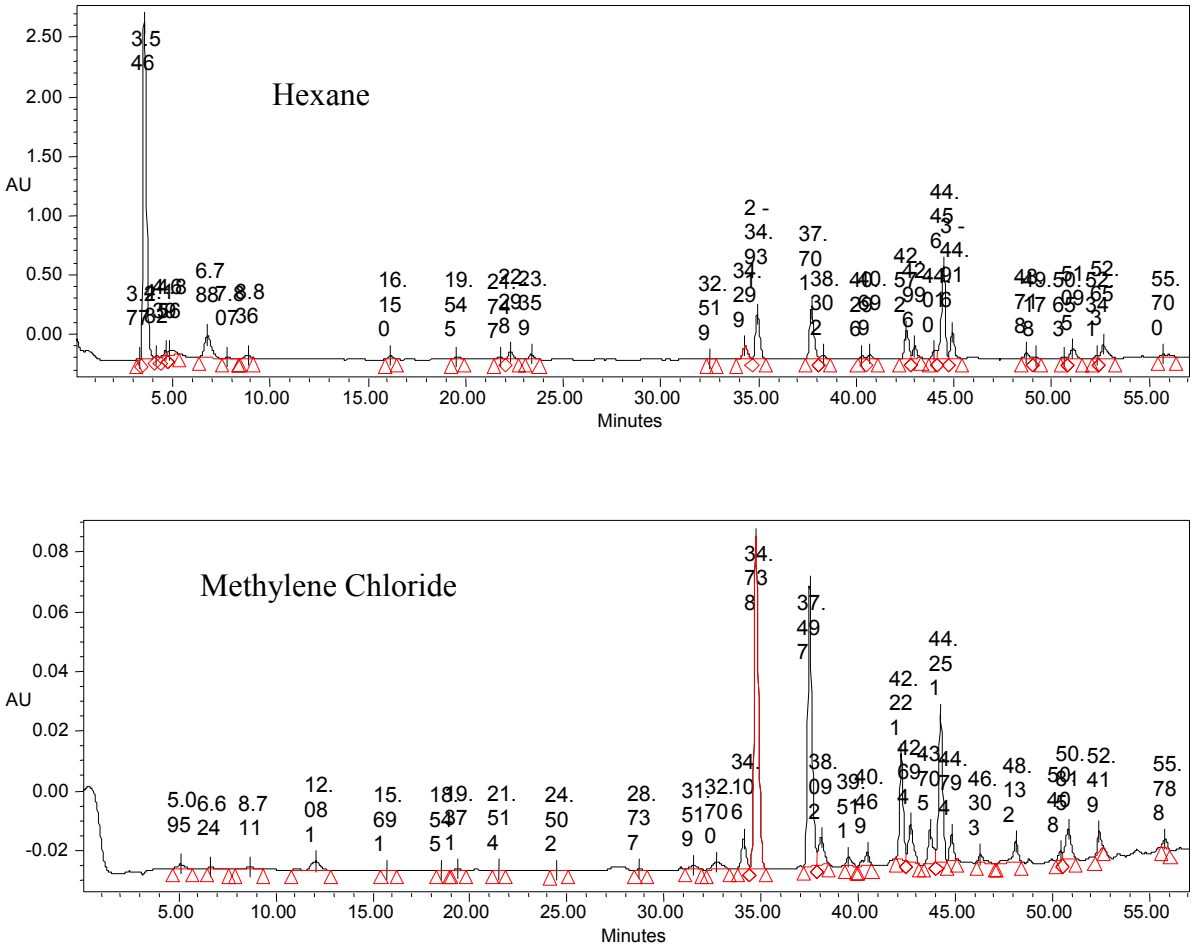


Figure 6. Chromatogram of Habanero extracts after HPLC analysis

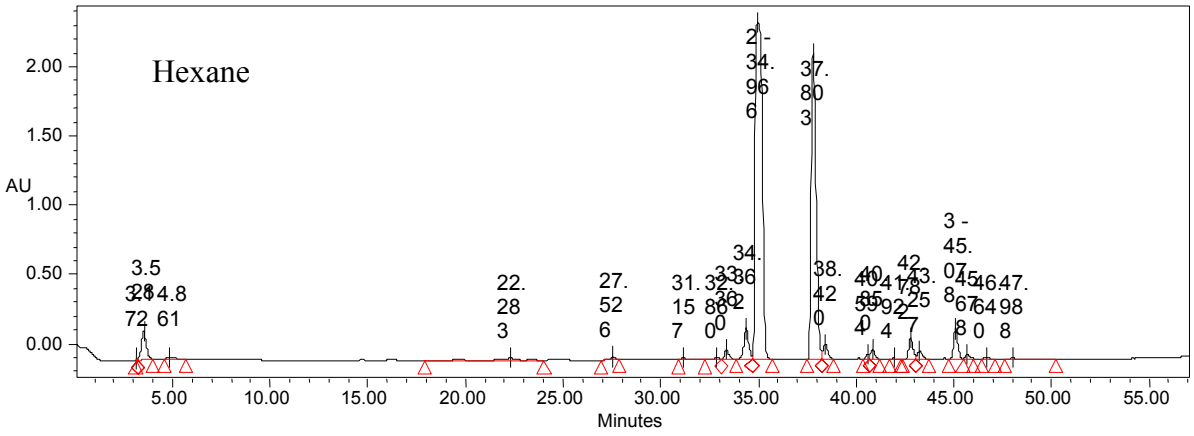


Figure 7. Chromatogram of Serrano extracts after HPLC analysis

CONCLUSIONS

The isolation of the biocidal agent or agents is progressing ahead of schedule. The addition of a fraction collector to the effluent from the HPLC will improve our capacity to isolate and test the peaks detected and reported in this report. The work on understanding the mechanisms and the modes of action of the extracted peppers is progressing well. The work is expanding on determining the activities of the extracts or fractionated components of the pepper extracts as quorum sensing inhibitors.

CITATIONS

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