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TARGETED LC-MS ANALYSIS FOR METABOLITE PROFILING FROM PATHOGENIC BACTERIA

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TARGETED LC-MS ANALYSIS FOR METABOLITE PROFILING FROM PATHOGENIC BACTERIA

by

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April 15, 2020

ABSTRACT

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Jessica Gomez-Vargas, B.S. Biological Chemistry

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Groundwater is relied on for drinking by more than 50 percent of the United States. For this reason, the purity of groundwater is extremely important. There has been possible contamination of opportunistic pathogenic microorganisms in groundwater attributable to oil and gas extractions from drilling and hydraulic fracturing; two of which are *Pseudomonas aeruginosa* and *Bacillus cereus*. These pathogens can cause severe infections in humans. *P. aeruginosa* increases prevalence of chronic and nosocomial infections. *B. cereus* produces toxins that are one of the most common causes of food poisoning. *B. cereus* can also cause infections of the eye, respiratory tract, and in wounds. Here, we quantitatively analyze possible metabolic therapies for these microorganisms by developing a metabolic profile for *P. aeruginosa* and *B. cereus* combining known secreted

or absorbed metabolites for these microbes with common metabolites found in nutrient and potato dextrose broth, through the use of liquid chromatography- mass spectrometry.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF TABLES

CHAPTER 1

INTRODUCTION

1.1 Contaminated Water

Groundwater is relied on for drinking by more than 50 percent of the United States. For this reason, the purity of groundwater is extremely important. Microbes play a significant role in groundwater quality by being used as biomarkers to determine contamination from fracking activities. Hydraulic fracturing involves the injection of high-pressure fluid into wells in order to fracture impermeable rocks, which eventually causes the release of trapped oil and gas. Natural gas production is increasing in the United States. Pollutants caused by hydraulic fracturing can greatly affect the amount of groundwater microorganisms. Despite the best attempts to prevent growth, microbes survive in fracking fluids due to the nutrients provided by the fracking additives.[1] There has been possible contamination of opportunistic pathogenic microorganisms in groundwater attributable to oil and gas extractions from drilling and hydraulic fracturing; two of which are *Pseudomonas aeruginosa* and *Bacillus cereus*.[2] In order to better understand these pathogens, their secreted and absorbed metabolites must be studied. Metabolites are substances that are crucial for the growth, development, and reproducibility of a particular organism. Some metabolites in bacteria can also be toxic and cause diseases to humans, classifying these bacteria as pathogens.

1.1.1 Pseudomonas aeruginosa

Pseudomonas aeruginosa is a gram-negative bacillus, typically found in nature, soil, and water. This bacterium is classified as an opportunistic pathogen that causes infection in patients with underlying or immunocompromising conditions like cancer, severe burns and cystic fibrosis. The genome of *P. aeruginosa*, has given scientists an understanding of the metabolic and pathogenic mechanisms of this versatile pathogen. *P. aeruginosa* has also become a representation for microbial genomic variation and evolution in chronic disease.[3]

Various metabolites were selected to be analyzed for this bacterium. These metabolites included: pyocyanin, rhamnolipids, 3-oxo-C12-homoserine Lactone, N-butyryl homoserine lactone, and cyclic-3'5'-diguanylic acid. Pyocyanin is a secondary secreted metabolite that functions by damaging host cells, tissues and immune system cells, inducing apoptosis, causing oxidative stress by mediating O_2 and H_2O_2 production. Rhamnolipid is also a secreted metabolite that causes cytotoxic, tissue invasion and damage, eliminate olymorphonuclear neutrophilic leukocytes, inhibit the mucociliary transport and ciliary function of human respiratory epithelium. This metabolite is also important for the maintenance of biofilm architecture and bacterial motility. 3-oxo-C12-homoserine and N-butyryl homoserine lactone are metabolites that are found to be added to media for *P. aeruginosa*. 3-oxo-C12-homoserine Lactone is linked to starvation, hypoxia, and oxidative stress. N-butyryl homoserine lactone is linked to oxidative stress. Lastly, cyclic-3'5'-diguanylic acid functions as a significant signal in post-transcriptional regulation of biofilm formation.[4]

1.1.2 Bacillus cereus

Bacillus cereus is a toxin-producing facultatively anaerobic gram-positive bacteria. This bacterium can be found in soil, vegetation, and foods. *B. cereus* causes two different types of intestinal illness: diarrhea and nausea/vomiting. This bacteria has also been observed to result in infections of the eye, respiratory tract, and in wounds.[5] Purine, uracil, and lactose were the chosen metabolites for this bacterium. *B. cereus* was found to secrete the metabolites purine and uracil. Lactose is a carbon fermentation compound that was discovered to display antimicrobial activity against *B. cereus*.[6]

1.1.3 Metabolites Taken From Selected Broths

In addition to the metabolites chosen for each pathogen, several other metabolites were analyzed because of their presence in two selected broths. Nutrient and potato dextrose broths were chosen for the reason that, both P. aeruginosa and B. cereus were successfully grown in these medias. Nutrient broth would be used as a maximal nutritional condition, whereas potato dextrose is a stressor. Both broths were ran through LCMS-8050 for the first time with an already developed method used for the monitoring of 96 compounds in media of prostate cancer cells and prostate cancer metastatic over 3 days, created by Jamie York, PhD in Schug Lab. This resulted in the addition of: arginine, citrulline, threonine, proline, adenine, guanosine, leucine, isoleucine, adenosine, xanthosine, pipecolinic, riboflavin, and fumaric acid.

1.2 Liquid Chromatograhy-Mass Spectrometry

Liquid chromatography - mass spectrometry (LC-MS) is an analytical technique that unites liquid chromatography separation abilities with mass spectrometry detection. LC-MS aims to detect and identify chemicals in the presence of other chemicals in a

mixture. Liquid chromatography is utilized to separate a sample into its individual components. The separation of analytes is possible due to their interactions between and with the mobile phase and stationary phase. Metabolites will have differences in their affinity for the stationary phase and mobile phase, causing the samples to elute from the column at different times, and be separated in a mixture.

LC can separate a wide range of organic compounds from small-molecule metabolites to peptides and proteins. Mass spectrometry (MS) offers is a sensitive detection technique that involves ionization of the sample components using various methods. The created ions are separated in vacuum based on their mass-to-charge ratio (m/z), the intensity of each ion can then be measured. MS detectors provide valuable information about molecular weight, structure, identity, quantity, and purity of the sample. MS detectors can be used for qualitative and quantitative analyses.

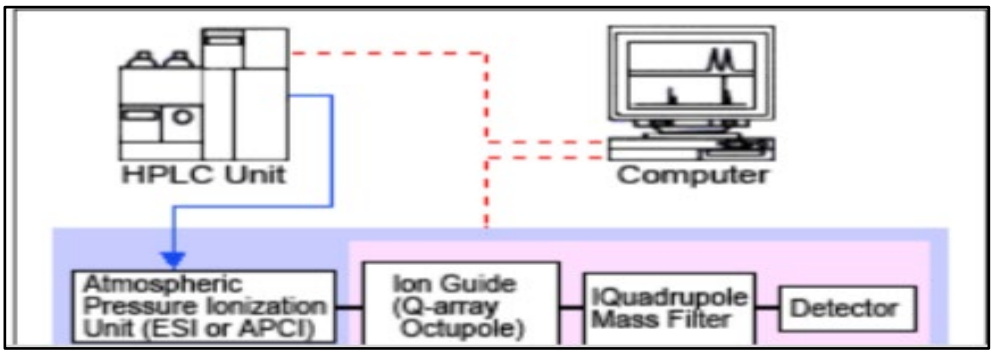


Figure 1.1: Liquid chromatography-mass spectrometry schematic[7]

LCMS is composed of a unit for separating components (HPLC), an ion source that ionizes the sample, an electrostatic lens that introduces the generated ions, a mass analyzer that separates ions based on their mass-to-charge ratio, and a detector unit that detects the separated ions.[7] This research will utilize a reversed phase LCMS technique, where the

stationary phase is nonpolar and the mobile phase is polar. Reverse phase LC-MS is commonly used for the analysis of polar analytes.

1.3 Mobile Phases

The mobile phase is the liquid that flows through a chromatography system, moving the materials to be separated at different rates over the stationary phase. The rate of movement of the analytes in the mobile phase is dependent on their interactions with the mobile and/or stationary phases. Due to differences in factors such as the solubility of certain components in the mobile phase and the strength of their affinities for the stationary phase, some components will move faster than others, thus resulting in the separation of the components within that mixture.[8] These are examples of mobile phase fluids used: water*, acetonitrile*, methanol*, ethanol, propanol, isopropanol, butanol, hexane, cyclohexane, toluene, and ethyl acetate. (*means these are the most common mobile phase solutions used).[7] Addition of modifiers in the mobile phase eluents can also enhance peak shape and increase sample load tolerance. Examples of mobile phase modifiers are: ammonium formate, formic acid, and ammonium acetate. The presence of formic acid improves the formation of protonated analyte ion resulting in a more uniform peak shape.[9]

1.4 Stationary Phases

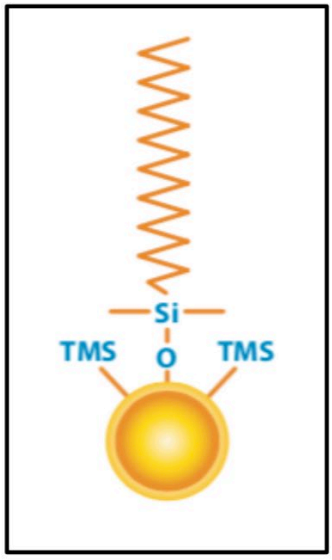
Separation of compounds by reversed-phase chromatography (RPC) relies on the reversible adsorption/desorption of the analytes, which have different degrees of hydrophobicity, to a hydrophobic stationary matrix (nonpolar). The hydrophobic binding interactions between the analyte and stationary phase is believed to be the cause of an entropy effect in areas near the hydrophobic regions where there is a greater degree of water structure. The matrices are typically silica-based (or synthetic organic polymers)

attached to covalently bound alkyl chains of different lengths such as *n*-octadecyl (C18), *n*-butyl (C4), or phenyl groups. The strength of the hydrophobic interaction increases as the alkyl chain size increases.[10]

Here is a series of column stationary phases (with their solute retention profile) utilized in this study:

1.4.1 C18

C18 structure:



Reversed phase retention interaction forces:

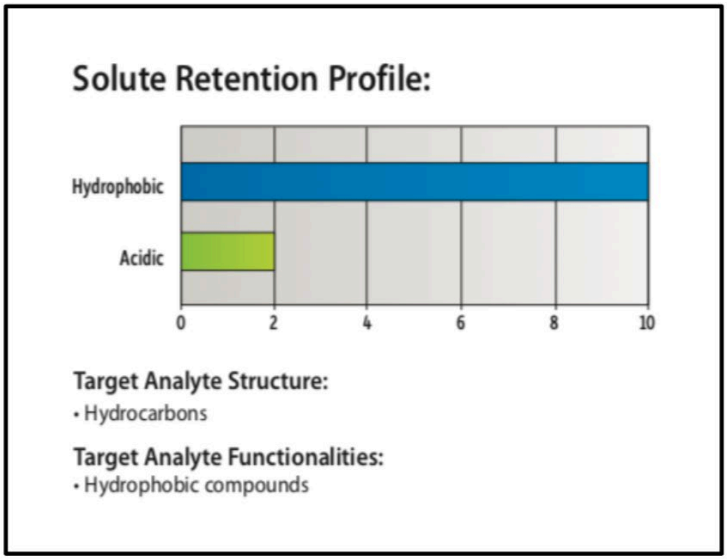
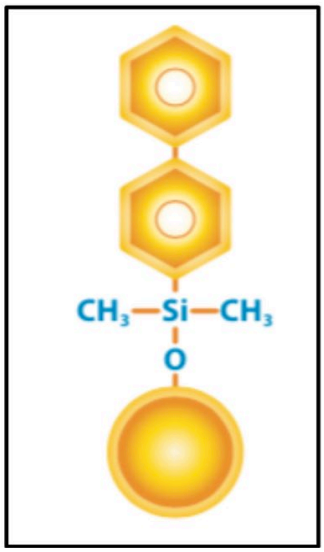


Figure 1.2: C18 column structure and solute retention profile[11]

1.4.2 Biphenyl

Biphenyl structure:



Reversed phase retention interaction forces:

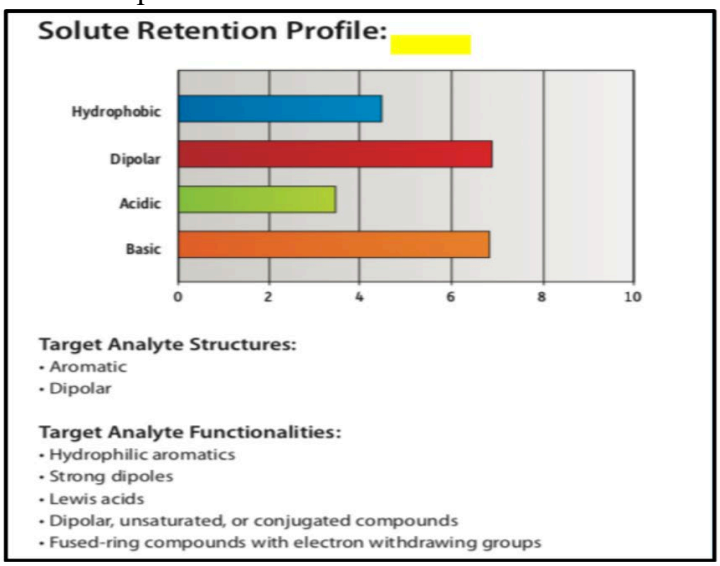
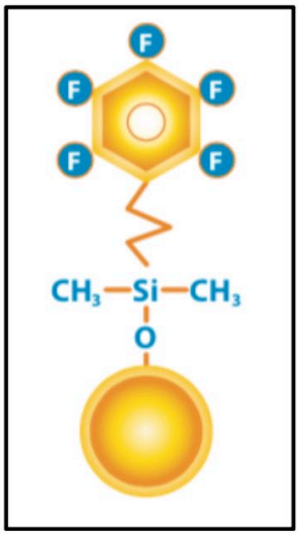


Figure 1.3: Biphenyl column structure and solute retention profile[11]

1.4.3 Pentafluorophenyl

Pentafluorophenyl structure:



Reversed phase retention interaction forces:

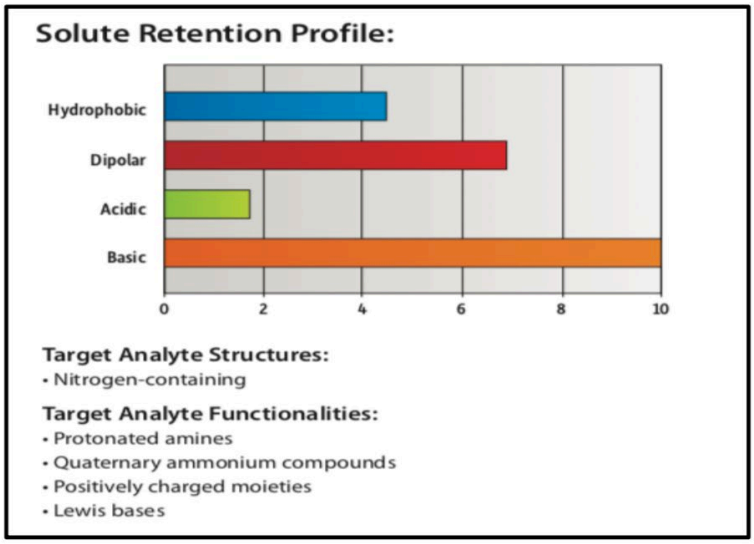


Figure 1.4: Pentafluorophenyl column structure and solute retention profile[11]

CHAPTER 2

LITERATURE REVIEW

2.1 Previous Metabolic Studies

Previous research has been conducted pertaining to the metabolites of *P. aeruginosa* and *B. cereus* that explain their pathogenicity, but no studies were shown to measure how these metabolites affect the microorganism's life cycle or how addition of other chemicals affect the secretion or absorption of certain metabolites in these pathogenic bacteria.

2.1.1 Pseudomonas aeruginosa Metabolites

Pseudomonas aeruginosa was previously found to have a secreted metabolite called pyocyanin in water and saliva through surface-enhanced Raman spectroscopy (SERS).[12] Pyocyanin is a virulence factor made by *P. aeruginosa* in bronchial secretions of cystic fibrosis patients, which plays a critical role for colonization and establishment of infections by *P. aeruginosa*. In addition to this molecule, rhamnolipids is also a compound that is a secreted virulence factor by this bacterium. Some examples of previously found metabolites in media for this bacterium are: 3-oxo-C12-homoserine Lactone, N-butyryl homoserine lactone, and cyclic-3'5'-diguanylic acid (cyclic di-GMP).[4]

In addition to this, scientists at Oxford University developed a Metabolome Database for this bacterium. This metabolome database provides more than 4370 metabolites and 938 pathways with links to over 1260 genes and proteins. The database information was obtained from electronic databases, journal articles and mass spectrometry

(MS) metabolomic data obtained in their lab. Each metabolite in the database contains detailed compound descriptions, names and synonyms, structural and physiochemical information, nuclear magnetic resonance (NMR) and MS spectra, enzymes and pathway information, as well as gene and protein sequences.[13]

Another group of scientists also reviewed mechanisms by which *P. aeruginosa* enhances its survival in various environments and particularly at different stages of pathogenesis. The illustrated the significance and complexity of regulatory networks and genotypic-phenotypic chances by which *P. aeruginosa* adjusts physiological processes for adaptation and survival as a result of environmental cues and stresses. The also studied the regulatory role of quorum sensing and signaling systems by nucleotide-based second messengers causing different lifestyles of *P. aeruginosa*.[4]

2.1.2 Bacillus cereus Metabolites

A quantitative and chemical assay of cereulide, the emetic toxin created in the cultures by some strains of Bacillus cereus was performed on a HPLC and a ESI electrospray ion trap mass analyzer, using the synthetic cereulide as a standard. All 20 strains of emetic *B. cereus* were found to secrete 27 - 740 ng/ml of cereulide by the LC-MS analysis. The bacteria was grown anaerobically at 37℃ on Luria-Bertani medium supplemented with 20mM K_3 PO_4 pH7, 2mM (NH_4)_2 SO_4, 1mM L-glutamic acid, 1mM L-tryptophan, 0.8mM L-phenylalanine, 0.005% (wt/vol) ammonium iron (III) citrate, 1mM glucose, 10mM nitrite. [14] In addition to this, the effect of *E. faecium* SM21 metabolites against B. cereus strains has also been previously studied. *L. johnsonii* CRL1647 secreted 128 mmol/L of lactic acid, 38 mmol/L of acetic acid and 0.3 mmol/L

of phenyl-lactic acid. These organic acids suppressed the number of vegetative cells and spores of the *B. cereus* strains tested.[15]

Additionally, the oxidative metabolism of *B. cereus* has been studied by Dr. Beck and Dr. Lindstrom. The following Table illustrates their findings on the uptake of some metabolites by *B. cereus*:[16]

Table 2.1: Oxidative and dehydrogenase activity of extracts of *Bacillus cereus*

Substrate	O_2 Uptake: µL in 60 Min*	Dehydrogenase Activity: Specific Activity†
Blank	25	0.1
Ethanol	156	3.3
Acetaldehyde	55	—
Pyruvate	53	—
Citrate	83	0.2
Isocitrate	—	0.5
α-Ketoglutarate	90	0.2
Succinate	100	0.2
Fumarate	463	2.8
Malate	366	2.8

2.1.2 Relevance and Extension of Previous Work

This project will focus on analyzing the twenty metabolites listed above. My study will be approached differently than the methods used in these previous studies for the reason that I will grow the bacteria in nutrient agar, and will analyze the collected data through triple quad reverse phase liquid chromatography- mass spectrometry (LCMS-8040) instead of HPLC, or SERS. Liquid chromatography separates the components of a sample based on their interaction with the stationary phase (nonpolar) or mobile phase (polar). Mass spectrometry ionizes the components of the sample; the ions are then separated in the vacuum based on their mass-to-charge ratio (m/z). The intensity of these ions can be used to determine the identity of the compounds in the sample.

CHAPTER 3

METHODOLOGY

3.1 MRM Optimization of Analytes

Optimization of multiple reaction monitoring (MRM) is an important part of any liquid chromatographic-mass spectrometric (LC-MS) method development. A typical MRM or dynamic MRM (dMRM) method contains two MS/MS transitions per analyte: one for quantification, and the second one is utilized for identification purposes. In a triggered MRM (tMRM) method using triple quadrupole LC/MS systems, up to 10 MS/MS transitions can be obtained for each analyte, and mixed into a product ion spectrum (at optimum collision energies for each product ion), which is used for library matching, and gives rise to increased identification confidence. [17]

3.1.1 Sample Preparation

In order to do MRM optimization for each analyte, a 1ppm concentrated solution must be made for each. This was done by weighing about 1 mg of each metabolite and dissolving it into 1 mL of their solvent. If the sample weight turned out to be more than 1 mg, for example 1.76 mg then it would get diluted with 1760 µL of solvent (which is equivalent to 1.760 mL). The following list gives each metabolite with its corresponding solvent: rhamnolipid (water), purine (water), pyocyanin (ethanol), N-butyryl homoserine lactone (DMSO), xanthosine (water), Adenine (water), arginine (water), citrulline (water), fumaric acid (water), isoleucine (water), pipecolinic (water), threonine (water), guanosine (water), proline (water), adenosine (water), lactose (water), leucine (water), 3-oxo-C12-

homoserine lactone (DMSO), cyclic-3'5'-diguanylic acid (water), Riboflavin (water). The 1 mg/ 1 ml solution will result in a final 1000 ppm concentration. This is too concentrated for the LC-MS since this instrumentation has a high sensitivity. Therefore 100 µL of this solution will be transferred to a new vial and 900 µL of LC-MS grade water is then added. These solutions are injected into LCMS-8040 for optimization.

3.2 Development of a Method

Once optimization of these analytes is completed, a new sample solution needs to be created combining all of the analytes listed above. In order to do this, 100 µL of the 1000ppm solution is transferred to a new vial where it will be diluted with 900 µL of LC-MS water resulting in a 100ppm solution. Then, 10 µL of each diluted metabolite samples are transferred to a new vial, 800 µL of LC-MS water is then added to make a 1ppm concentration of each analyte in solution. This mixture can then be successfully utilized after vortex, for metabolic profiling method development.

3.2.1 Mobile Phase Solvents

Many different mobile phase solvents were utilized for in this research. The following mobile phase B (which is considered the most polar solvent) were tested: Acetonitrile, methanol, methanol with 0.1% formic acid, methanol with 10 mM ammonium formate, methanol with 10 mM ammonium acetate. The best solvent was found to be methanol with 0.1% formic acid. The mobile phase A (less polar solvent) was composed of water with 0.1 % formic acid. In order to create these solvents, 1000 µL of formic acid was added to 1 L of water/ methanol. Most of these methods had these set parameters: Injection volume is 5 µL, flow rate: 0.4mL/min, column oven temperature: 40℃, nebulizing gas flow: 2L/min, drying gas flow: 15L/min, DL temperature: 250℃, Heat

Block Temperature: 400°C. A 2-3 minute re-equilibrium time is done at the end of each method to go back to initial starting conditions. The internal standard chosen was 2-isopropylmalic acid.

3.2.2 5 to 100% B PFP Column

The first method development was with a pentafluorophenyl stationary phase. During this time, I only had 8 compounds to study. The mobile phase conditions were as followed: starting conditions were set to 5% B concentration, 12:00min (100%), 15:00min (100%), 15:01min (5%), 18:00min (stop). The values in the parenthesis symbolize solvent B concentration (polar mobile phase).

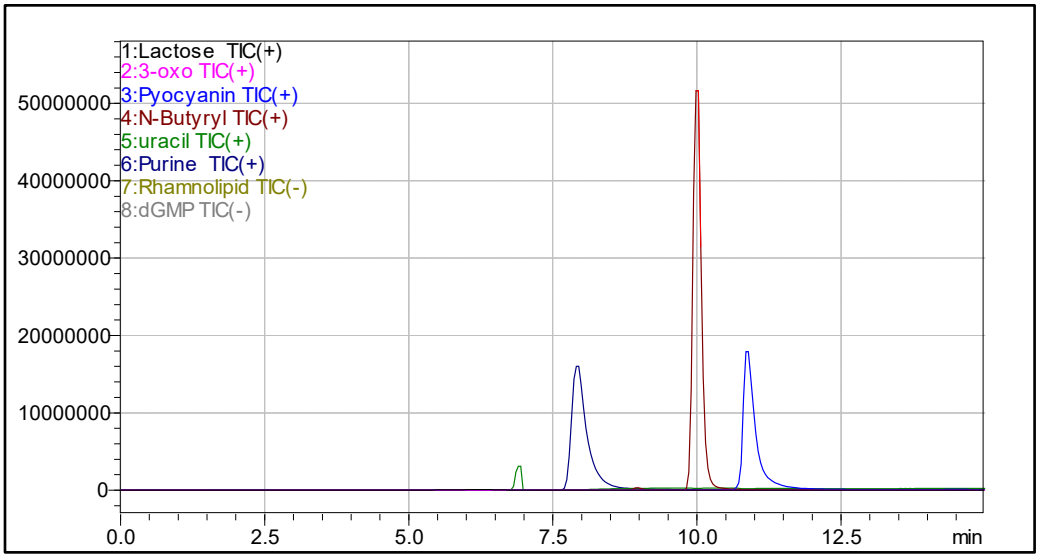


Figure 3.1: Chromatogram of 5 to 100% B PFP column method

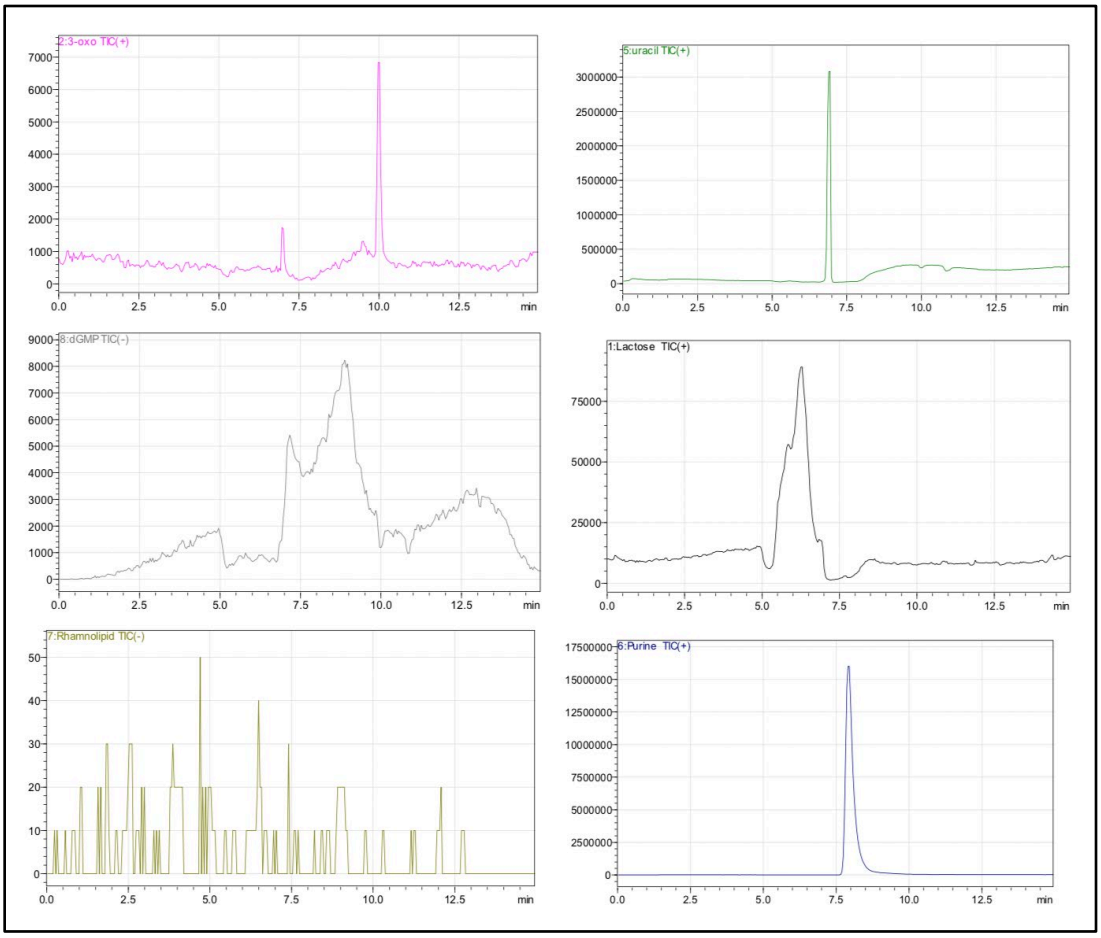


Figure 3.2: Single peaks of analytes from a 5 to 100% B with PFP column method

3.2.3 Holding 100% B longer on Biphenyl Column

The second method development was with a biphenyl stationary phase. During this time, I only had 8 compounds to study. The mobile phase conditions were as followed: starting conditions were set to 5% B concentration, 8:00min (100%), 12:00min (100%), 15:00min (100%), 15:01min (5%), 18:00min (stop).

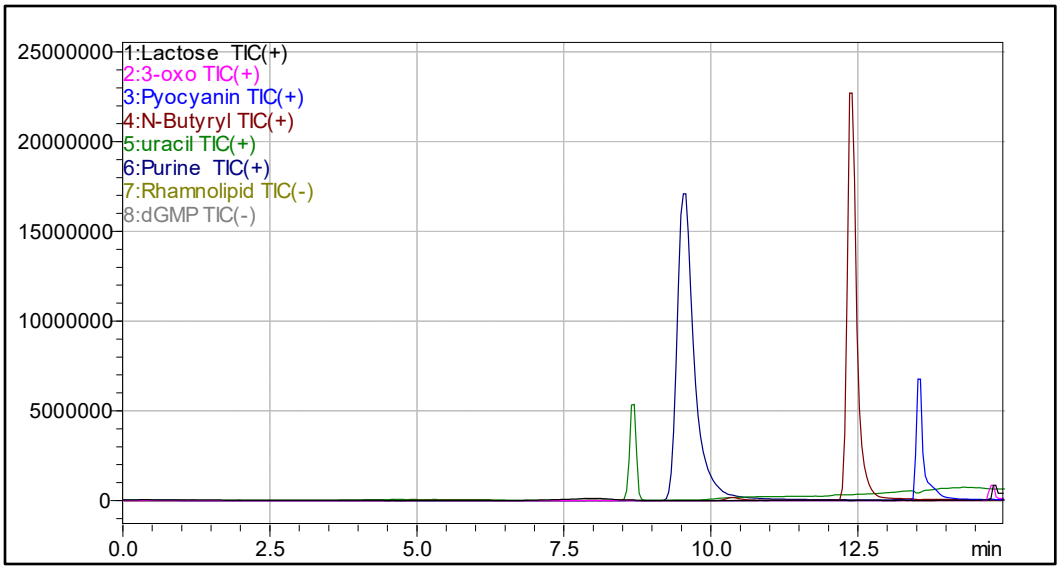


Figure 3.3: Chromatogram of holding 100% B longer on biphenyl column method

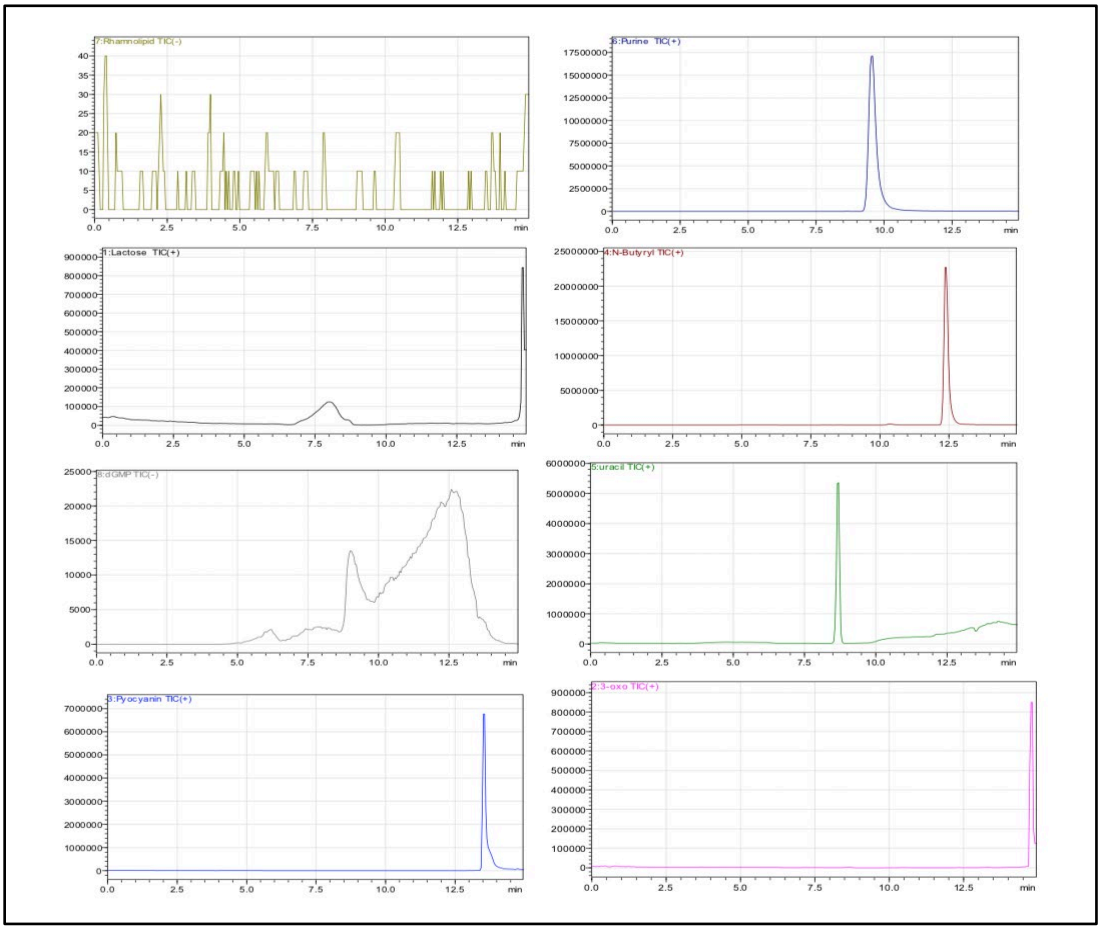


Figure 3.4: Single peaks of analytes from a holding 100% B with biphenyl column method

3.2.4 50 to 100% B on Biphenyl Column

The third method development was with a biphenyl stationary phase. During this time, I only had 8 compounds to study. The mobile phase conditions were as followed: starting conditions were set to 50% B concentration, 4:00min (100%), 6:00min (100%), 7:30min (100%), 7:31min (50%), 9:00min (stop).

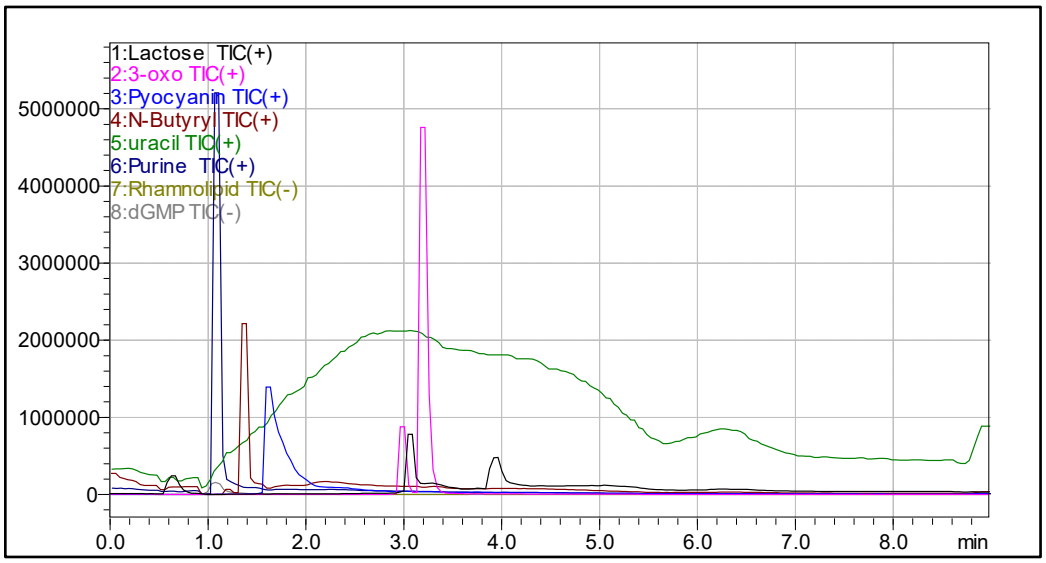


Figure 3.5: Chromatogram of 50 to 100% B on biphenyl column method

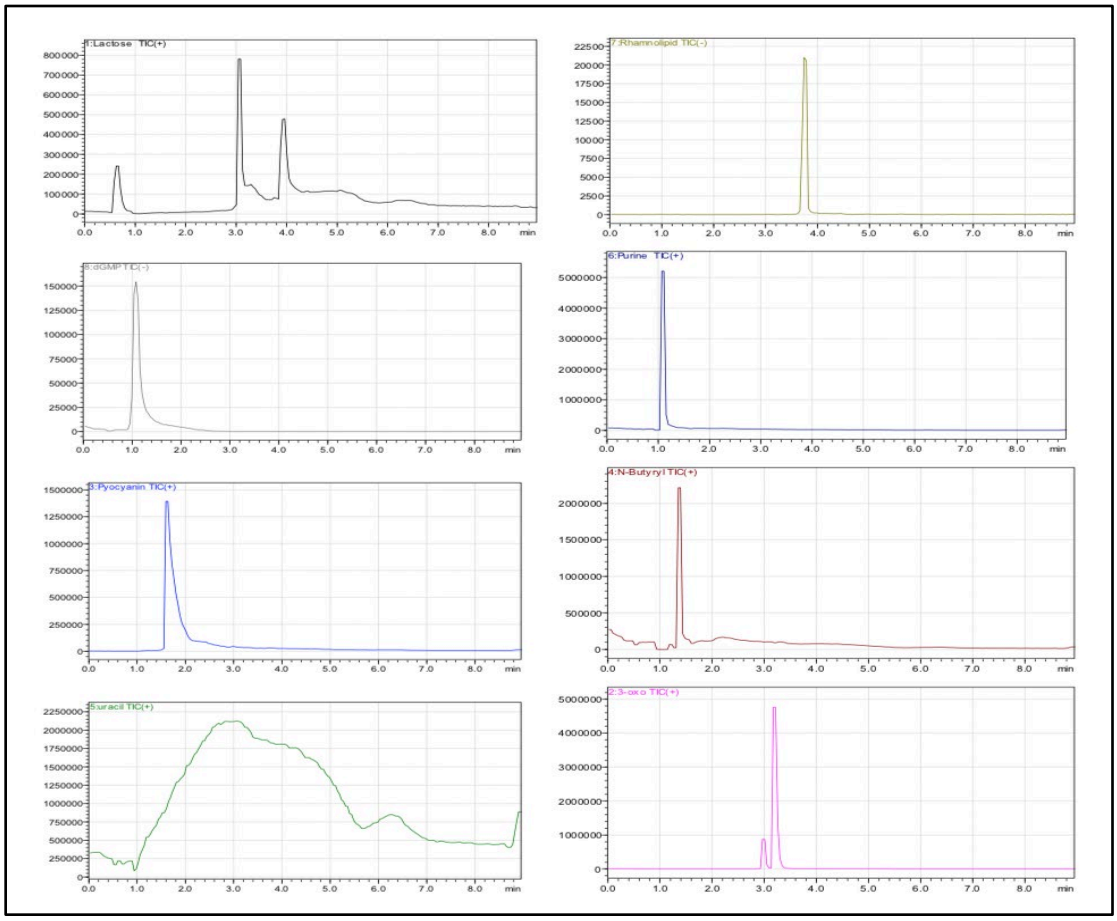


Figure 3.6: Single peaks of analytes from a 50 to 100% B with biphenyl column method

3.2.5 5 to 100% B on Biphenyl Column

The fourth method development was with a biphenyl stationary phase. During this time, I only had 8 compounds to study. The mobile phase conditions were as followed: starting conditions were set to 5% B concentration, 8:00min (100%), 12:00min (100%), 15:000min (100%), 15:01min (100%), 18:00min (stop).

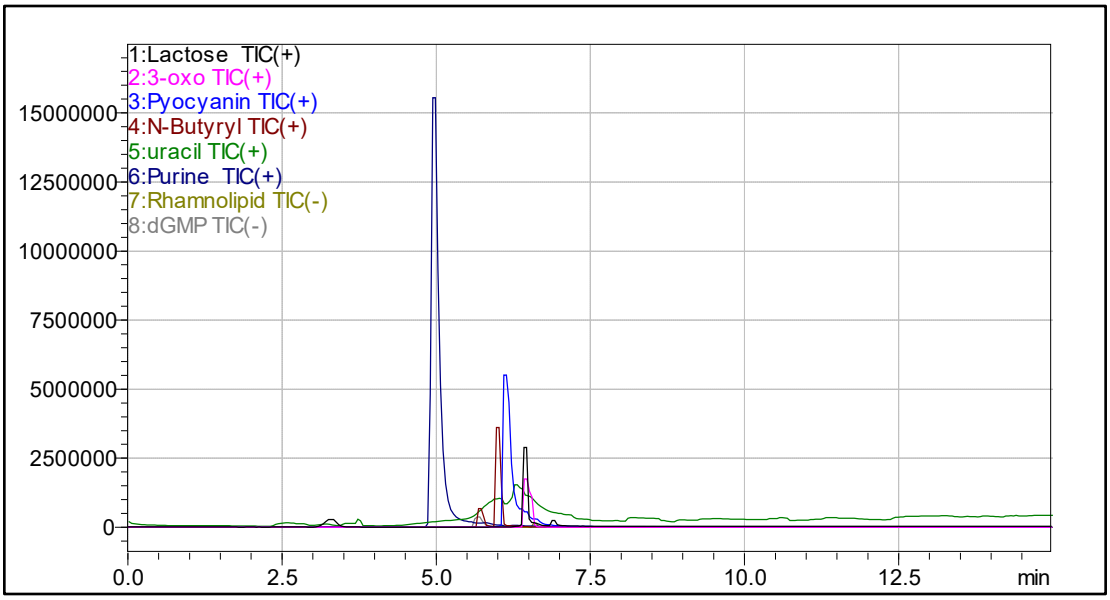


Figure 3.7: Chromatogram of 5 to 100% B on biphenyl column

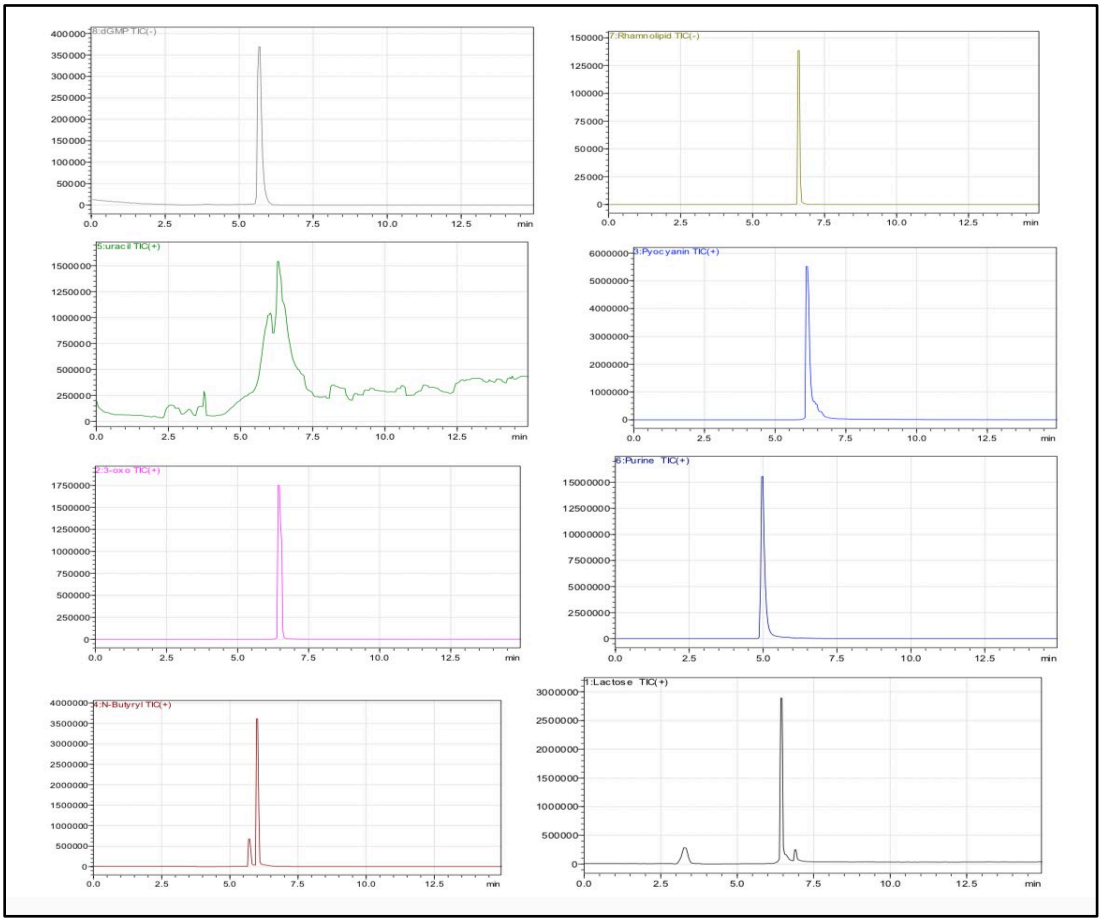


Figure 3.8: Single peaks of analytes from a 5 to 100% B with biphenyl column method

3.2.6 20 compounds on PFP Column

The fifth method development was with a pentafluorophenyl stationary phase. During this time, I was analyzing 20 compounds. The mobile phase conditions were as followed: starting conditions were set to 0% B concentration, 2:00min (0%), 8:00min (90%), 13:000min (90%), 13:01min (0%), 15:00min (stop).

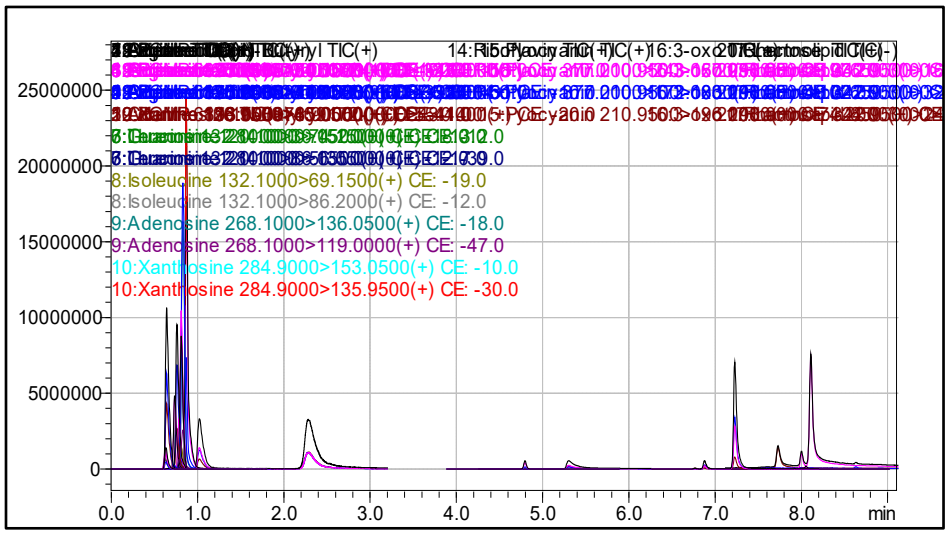


Figure 3.9: Chromatogram of 20 compounds on PFP column

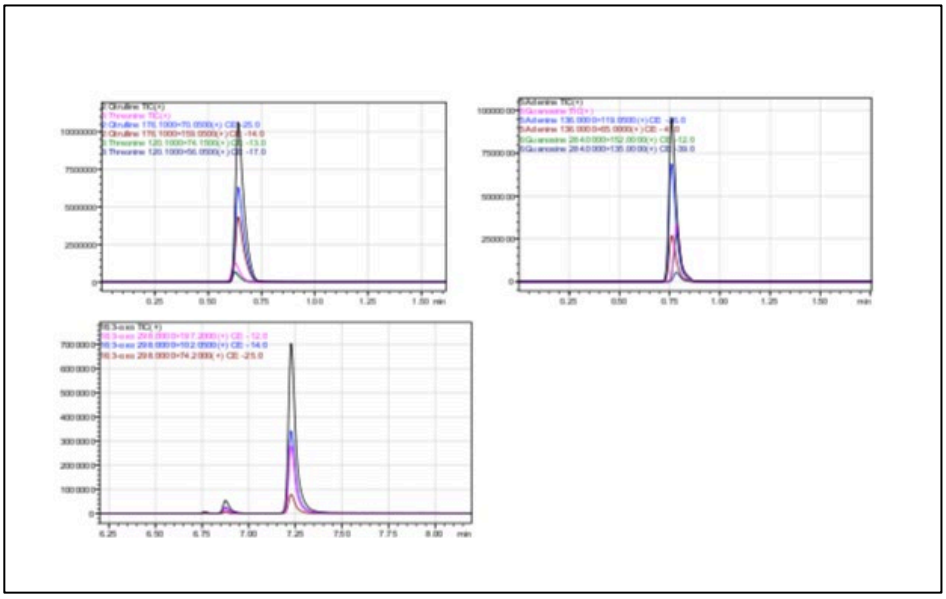


Figure 3.10: Single peaks of analytes from a 0 to 90% B with PFP column method

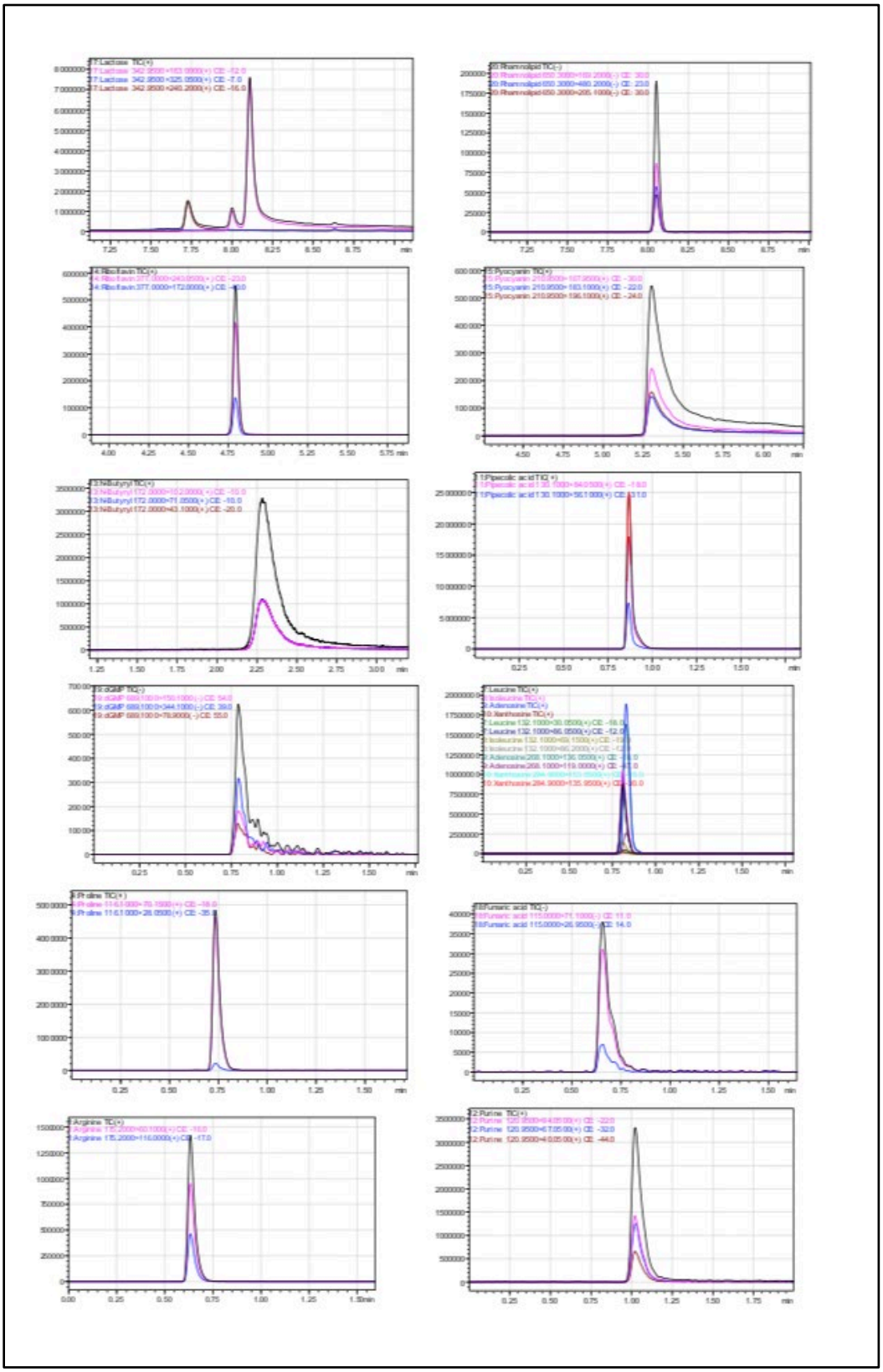


Figure 3.11 Single peaks of analytes from a 0 to 90% B with PFP column method (continued)

3.2.7 20 compounds on PFP Column in Different Solvents

The sixth method development was with a pentafluorophenyl stationary phase. During this time, I was analyzing 20 compounds. This time I separated the analytes into the following categories: neutrals, basics, and DMSO soluble. The solvent for basic analytes, which was 1N NaOH was constructed by dissolving 4 grams of sodium hydroxide in 10 mL of LC-MS grade water. The mobile phase conditions were as followed: starting conditions were set to 0% B concentration, 2:00min (0%), 9:00min (100%), 19:000min (100%), 19:01min (0%), 21:00min (stop).

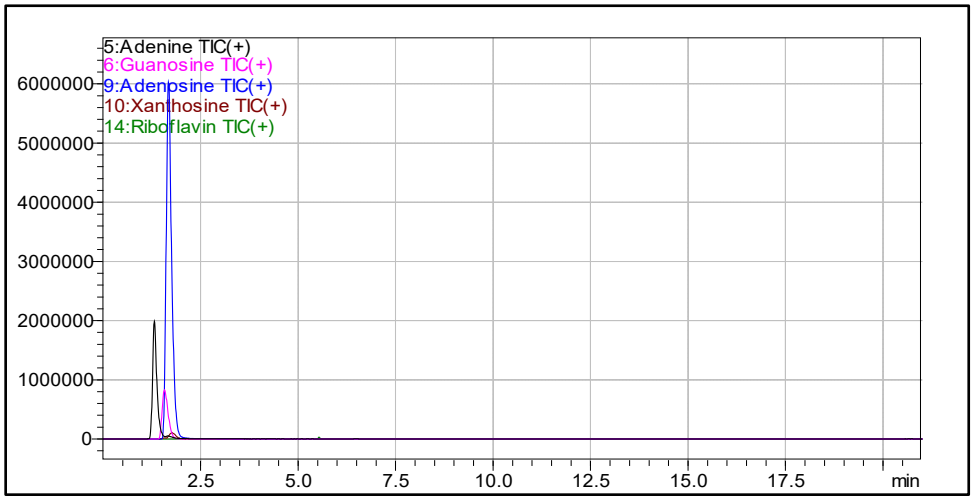


Figure 3.12: Chromatogram of basic analytes dissolved in 1N NaOH

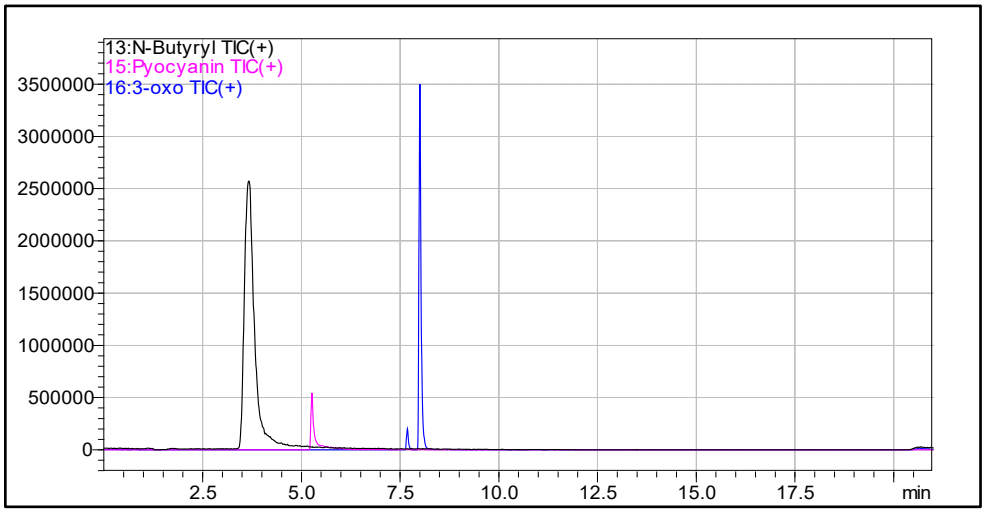


Figure 3.13: Chromatogram of DMSO soluble analytes

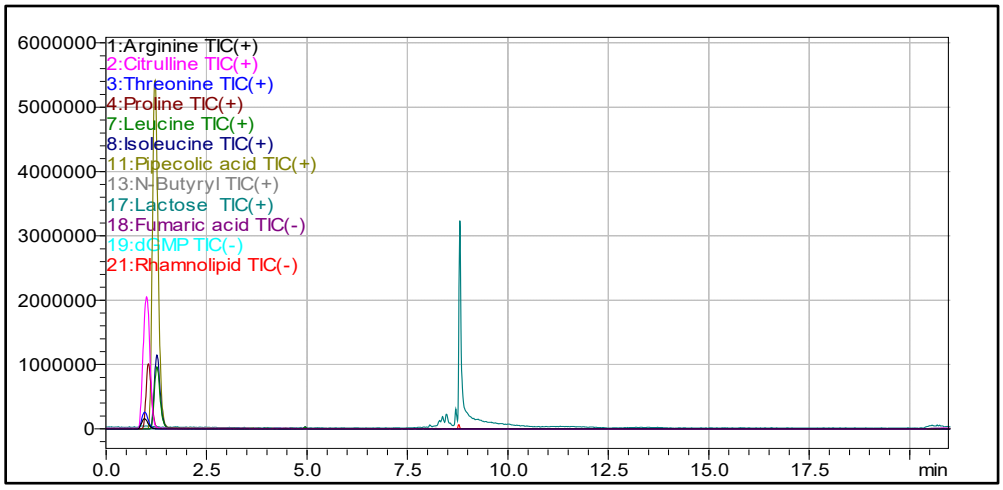


Figure 3.14: Chromatogram of neutral analytes dissolved in LC-MS grade water

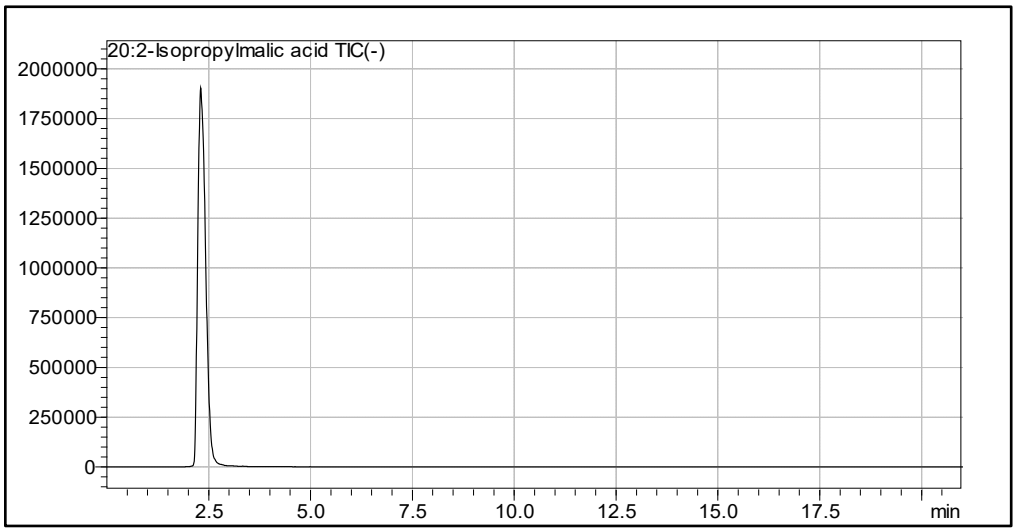


Figure 3.15: Chromatogram of internal standard

3.2.8 Verification of Reproducibility

In order to confirm that the above method resulted yielded the best results meaning that it is reproducible, the standard deviations and percent of relative standard deviations for retention times and areas of 5 sequential runs were calculated. The following tables illustrate these calculated values for each metabolite.

Table 3.1: Average, standard deviation, and percent standard deviation values for the retention times of neutral metabolites

Metabolites	Retention Times- Neutrals				Average	STD DV	% RSD
Arginine	0.958	0.952	0.955	0.965	0.958	0.00572	0.60
Threonine	0.963	0.942	0.96	0.95	0.954	0.00960	1.01
Citrulline	0.996	1.009	1.003	1.007	1.00	0.00574	0.57
Proline	1.052	1.053	1.048	1.058	1.053	0.004113	0.39
Fumaric Acid	1.196	1.18	1.201	1.212	1.197	0.01330	1.11
Pipecolic Acid	1.213	1.217	1.217	1.213	1.215	0.002309	0.19
Isoleucine	1.259	1.265	1.265	1.274	1.266	0.006185	0.49
Leucine	1.261	1.264	1.25	1.266	1.260	0.007136	0.57
dGMP	1.58	1.606	1.661	1.65	1.624	0.03788	2.33
Purine	1.885	1.877	1.881	1.886	1.882	0.004113	0.22
Rhamnolipid	8.781	8.777	8.778	8.781	8.779	0.002062	0.02
Lactose	8.805	8.803	8.804	8.804	8.804	0.0008165	0.01

Table 3.2: Average, standard deviation, and percent standard deviation values for the area of peaks of neutral metabolites

Metabolites	Area of Peaks- Neutrals				Average	STD DV	% RSD
Arginine	1245582	1191757	1167458	1174837	1194908.5	35280.451	3.0
Threonine	1452102	1426266	1445901	1440147	1441104	11031.01	0.77
Citrulline	14631057	13553769	12949342	13002531	13534174.75	780642.6898	5.8
Proline	8946984	8968628	8937486	8869306	8930601	42891.086	0.48
Fumaric Acid	127211	123744	126367	128847	126542.25	2130.7287	1.7
Pipecolic Acid	36932071	36851712	36130647	36270521	36546237.75	404523.2634	1.1
Isoleucine	9658523	9849838	9880308	10016729	9851349.5	147618.11	1.5
Leucine	8624784	9229461	9093306	9072316	9004966.75	262853.785	2.9
dGMP	31232	46306	47605	84139	52320.5	22476.3	43.0
Purine	17015489	16137672	16036571	15712342	16225518.5	557022.344	3.4
Rhamnolipid	81651	73154	66719	64534	71514.5	7684.63	10.7
Lactose	15831121	16042393	14290062	15016532	15295027	802800.69	5.2

Table 3.3: Average, standard deviation, and percent standard deviation values for the retention times of basic metabolites

Metabolites	Retention Times- Basics					Average	STD DV	% RSD
Adenine	1.319	1.306	1.312	1.309	1.308	1.3108	0.0050695	0.39
Guanosine	1.568	1.569	1.573	1.579	1.574	1.5726	0.0043932	0.28
Adenosine	1.681	1.679	1.676	1.675	1.678	1.6778	0.0023875	0.14
Xanthosine	1.768	1.774	1.766	1.781	1.768	1.7714	0.0061482	0.35
Riboflavin	5.535	5.53	5.537	5.531	5.532	5.533	0.002915	0.053

Table 3.4: Average, standard deviation, and percent standard deviation values for the area of peaks of basic metabolites

Metabolites	Area of Peaks- Basics					Average	STD DV	% RSD
Adenine	10972041	10769736	10700904	10598033	10447806	10697704	195475.67	1.8
Guanosine	7528493	7432615	7463068	7324154	7395668	7428799.6	76118.426	1.0
Adenosine	50783497	50028170	49632792	48901492	47824877	49434165.6	1127667.93	2.3
Xanthosine	1182143	1172738	1107606	1117174	1132698	1142471.8	33320.327	2.9
Riboflavin	70255	72642	72245	66855	69724	70344.2	2316.25	3.3

Table 3.5: Average, standard deviation, and percent standard deviation values for the retention times of DMSO soluble metabolites

Metabolites	Retention Times- DMSO Soluble					Average	STD DV	% RSD
N-Butyryl	3.648	3.665	3.647	3.642	3.674	3.6552	0.013627	0.37
Pyocyanin	5.266	5.27	5.27	5.27	5.266	5.2684	0.0021909	0.042
3-oxo	7.999	7.999	8	7.998	8	7.9992	0.00083666	0.010

Table 3.6: Average, standard deviation, and percent standard deviation values for the area of peaks of DMSO soluble metabolites

Metabolites	Area of Peaks- DMSO Soluble					Average	STD DV	%RSD
N-Butyryl	15452634	15140482	14887295	14786284	14400006	14933340.2	393943.153	2.6
Pyocyanin	1386894	1194653	1161543	1113044	1037994	1178825.6	130386.36	11
3-oxo	6531668	6409309	6089891	6216389	6226799	6294811.2	174627.37	2.8

Table 3.7: Average, standard deviation, and percent standard deviation values for the retention times and area of peaks for the internal standard

Internal Std	Retention Times					Average	STD DV	%RSD
2-isopropylmalic Acid	2.315	2.304	2.3	2.305	2.304	2.3056	0.0055946	0.24
Internal Std	**Area of Peaks**					**Average**	**STD DV**	**%RSD**
2-isopropylmalic Acid	15543166	15804438	16217520	16516869	16187570	16053912.6	381464.276	2.4

CHAPTER 4

DISCUSSION

Several different methods were studied in order to develop a metabolic profile for pathogenic bacteria *B. cereus* and *P. aeruginosa*. The first four methods developed did not produce the best peak shapes. When observing the first method where a 5 to 100% mobile phase ramp was utilized with a pentafluorophenyl column, the chromatogram (shown in figure 3.1) can be quickly glanced at and seen that only a few compounds were able to be successfully retained. No retention was acquired with rhamnolipid, and this could be due to the usage of just plain water and methanol as the mobile phase solvents. Figure 3.2 also shows that dGMP and lactose were not properly isolated. Both of these metabolites had a peak shoulder; this is due to a void in the column inlet or the analyte having to take multiple paths.

In the second attempt for method development, I held 100% mobile phase B for a longer time on a biphenyl column. Figure 3.3 shows that the well retained samples were eluded out at later times (past 8 minutes). Figure 3.4 shows that rhamnolipid was still not able to be retained. This figure also shows that lactose and 3-oxo eluded right before the method ended. This shows that the mobile phase B would have needed to be extended for a longer time in order to retain these metabolites earlier in the chromatogram. dGMP had a broad peak, this is the cause of incompletely filled sample loops, incompatibility of the injection solvent with the mobile phase, or poor sample solubility.

In order to shift the analytes to the left on the chromatogram, the third method started off at 50% mobile phase B instead of 5%. Figure 3.5 shows that I was able to shift the analytes to the left but there was still poor retention on some metabolites. Figure 3.6 shows that this was the case for uracil. I had to remove uracil after trying to incorporate it into this analysis for about 6 months. I failed to develop a successful method in the beginning with uracil because it typically elutes out in the dead time of revere phase liquid chromatography. The dead time occurs when one column volume worth of mobile phase passes through the column. Typically uracil can be retained well in normal phase chromatography, not reverse phase.

Next, the 5 to 100% B in biphenyl method was tried again but with a 0.1% formic acid modifier in both mobile phase A and B eluents. Due to this, more analytes were able to be retained and this is seen in figure 3.8. The only analyte that was not well retained was uracil once again. Once I added more compounds to my list of metabolites a 0 to 90% B method was developed with a pentafluorophenyl stationary phase. Figure 3.9 illustrates the chromatogram for this method. It is observed to have an even distribution of analytes across the graph. Figure 3.10 and 3.11 show great uniform peaks for each one of the compounds.

This method was further enhanced by the separation of compounds into neutral, basic, and dmso soluble categories. Figues 3.12 – 3.15 will display the excellent separation of these metabolites in mixture. In order to confirm that this method is effective and reproducible, the averages, standard deviations, and percent of relative standard deviations (% RSD) of 5 separate runs with the same method were calculated. If percent relative standard deviations are above 10% that means results are unreliable. This was only seen for three occasions. Table 3.2 has 2 red cells, marking the high % RSD values for dGMP

and rhamnolipid. In addition to this, table 3.6 has a red cell pertaining to pyocyanin's 11% RSD value for its area peak. All of these metabolites have a high % RSD for the reason that, the original 1000ppm stock solutions were made around 9 months ago and were not stored in the refrigerator. Only a small amount of analytes were ordered through Sigma Aldrich due to their high cost. Therefore, their high % RSD is tied to metabolic degradation. As a result, qualitative analysis (determines if the metabolite is present) can be done for these analytes instead of quantitative analysis (how much of it is there).

The final method was extended from 15 minutes to 21 minutes and ramped up the mobile phase B from 90 to 100% because of metabolic contamination of lactose in column. Lactose was beginning to build up in the column and produced a large peak in the chromatogram. Different wash techniques were employed in order to clean the column and elude all of the lactose out. Several blank injections were done, this causes the injector to not take any of the sample solution to the MS and just run the column with mobile phase solvents. The column was also washed out with 100% methanol for 3 hours, this did not help and there was still lactose saturation present in the column. Isopropyl alcohol was also taken from the injector in order to do an injector clean, the problem was not fixed meaning that the injector was not contaminated. Lastly, I washed the column with acetonitrile for 1 hour (which was a stronger solvent than the most polar mobile phase in my method) and it resulted in the successful clean out of lactose. In order to avoid this problem the most polar solvent was held at 100% for a longer time.

CHAPTER 5

CONCLUSION

The metabolic profiles of *P. aeruginosa* and *B. cereus* were successfully created. This is significant for the reason that, there has been an alarming upbringing of possible contamination in groundwater with these opportunistic pathogenic microorganisms due to oil and gas extractions because of drilling and hydraulic fracturing. The major focus of this study was to develop a metabolic profile for these bacteria to quantitatively analyze metabolites that are taken up by these two microorganisms or secreted.

5.1 Future Studies

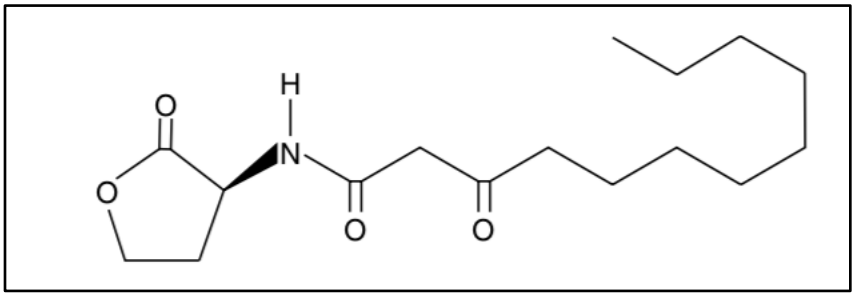
This research can be further expanded by analyzing how the addition of targeted chemicals affect proliferation or cell death of bacteria through liquid chromatography mass spectrometry (LMCS-8040). In addition to this, possible metabolic therapies for these microorganisms can be examined by evaluating how different concentration of selected compounds affect the absorption or secretion of certain toxic metabolites for *P. aeruginosa* and *B. cereus* through the use of liquid chromatography- mass spectrometry. This is approached by incubating the bacteria with different compounds and observe modulation of the already developed metabolic profile, in the context of the above-listed metabolite targets. For example, *P. aeruginosa* could be incubated with different concentrations of 2-ketobutyric acid, known to degrade threonine. Whereas, *B. cereus* could be grown with lactic acid, known to reduce vegetative cells and spores. The findings in this study will aid

in future development of medications to alter the secretion of certain toxic metabolites in the studied pathogenic bacteria.

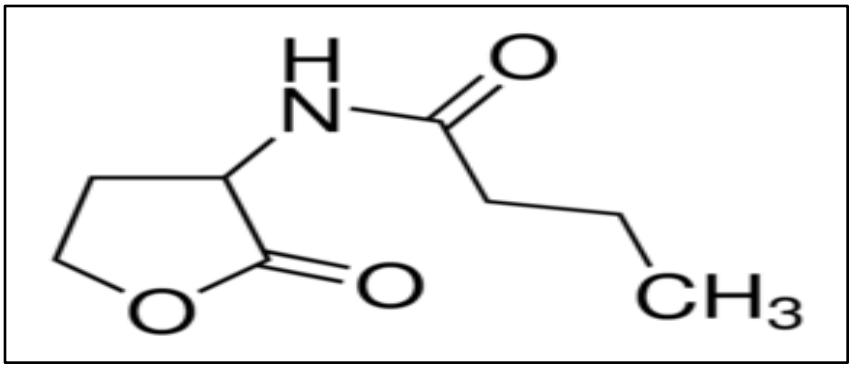
APPENDIX A

METABOLIC CHEMISTRIES

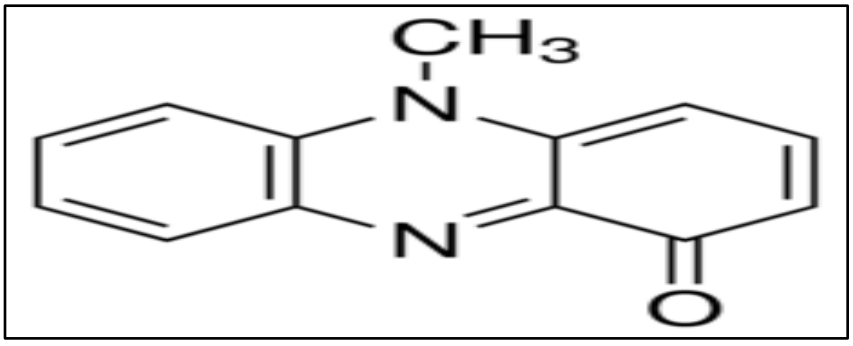
Name: 3-oxo-C12-homoserine Lactone



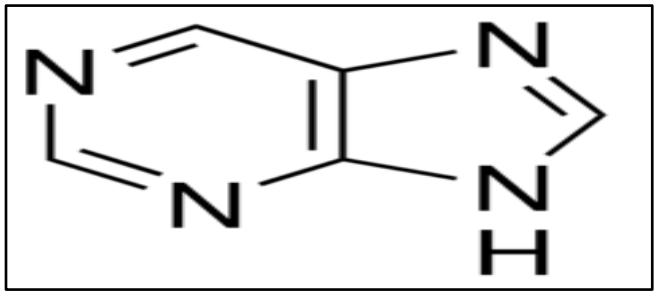
Name: N-Buyryl homoserine lactone



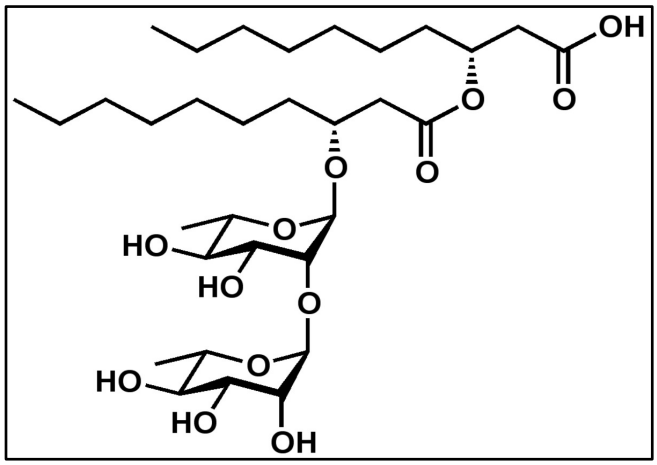
Name: Pyocyanin



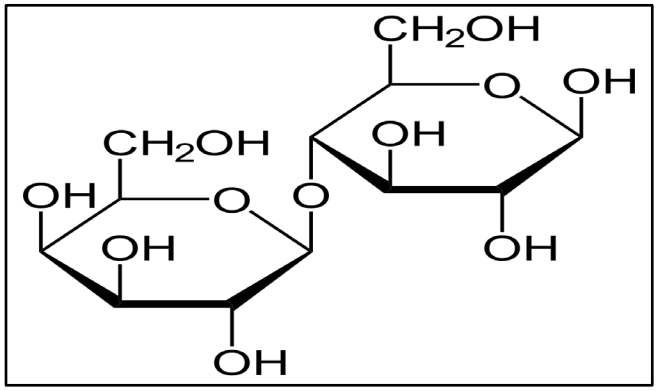
Name: Purine



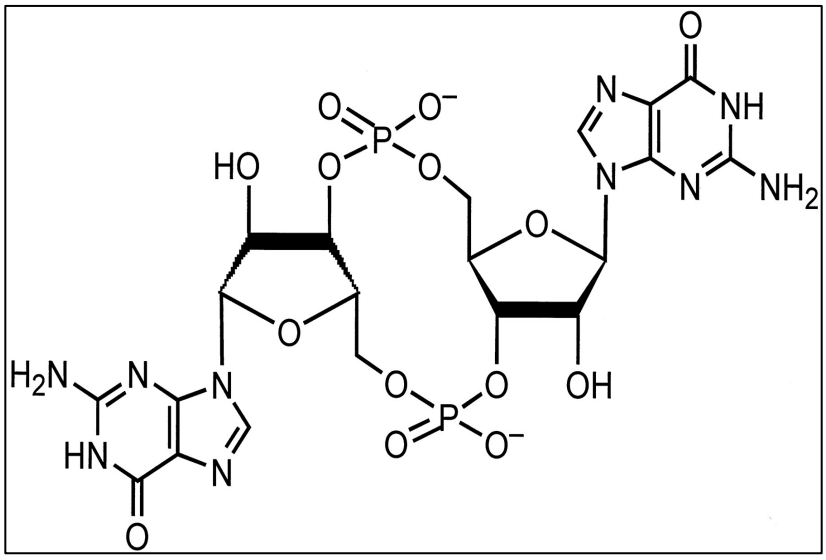
Name: Rhamnolipids



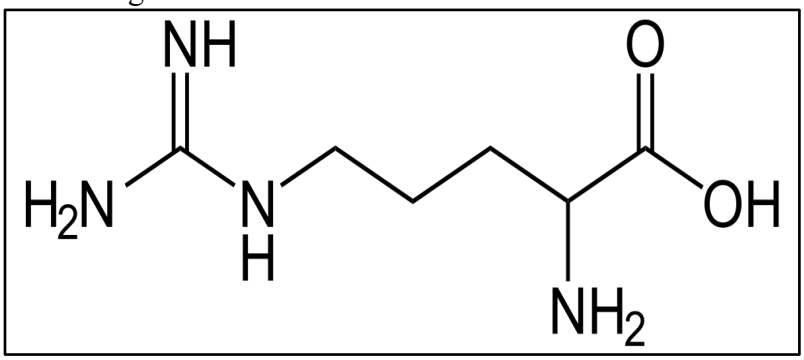
Name: Lactose



Name: Cyclic-3’5’-diguanylic acid



Name: Arginine



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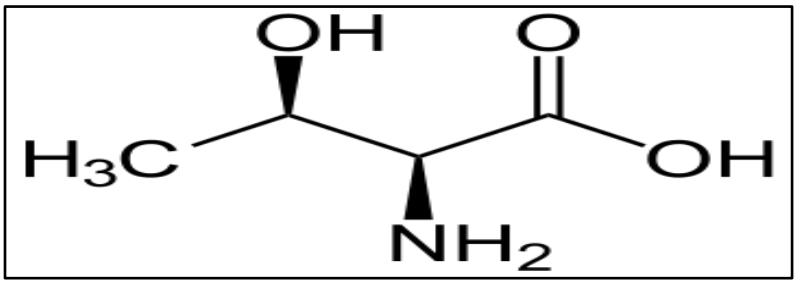
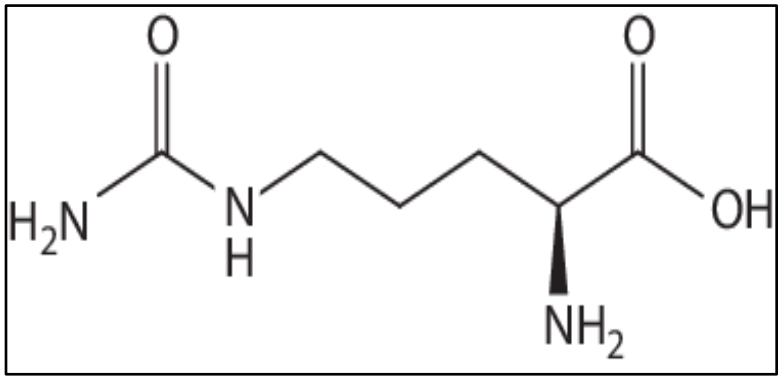
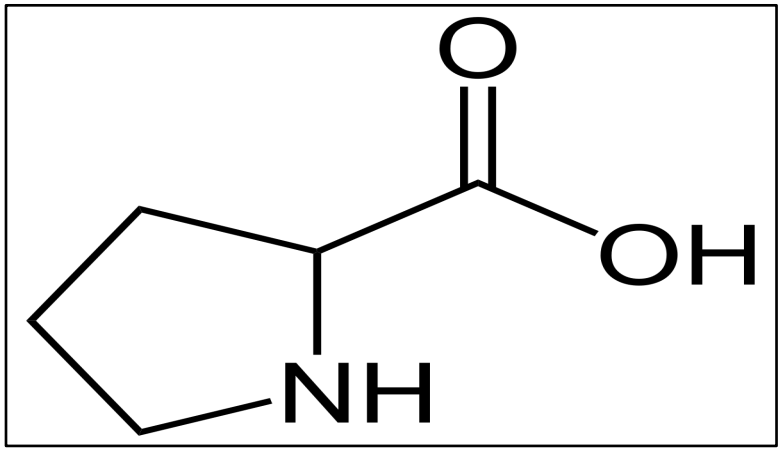


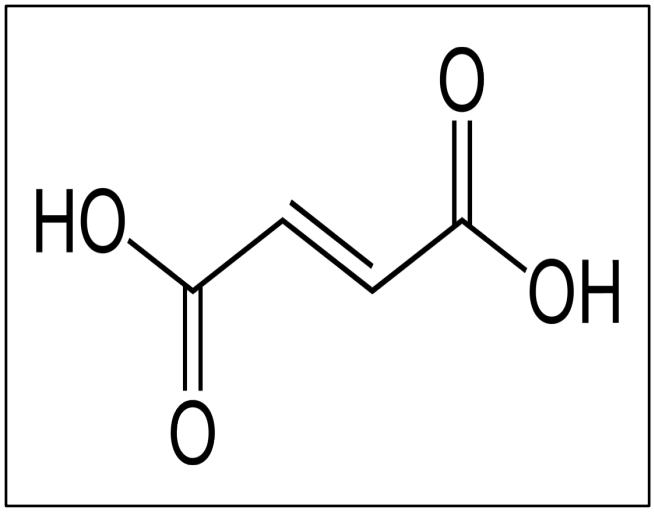
Name: Citrulline



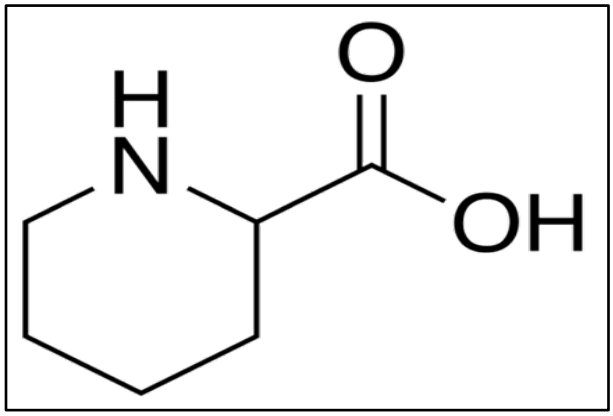
Name: Proline



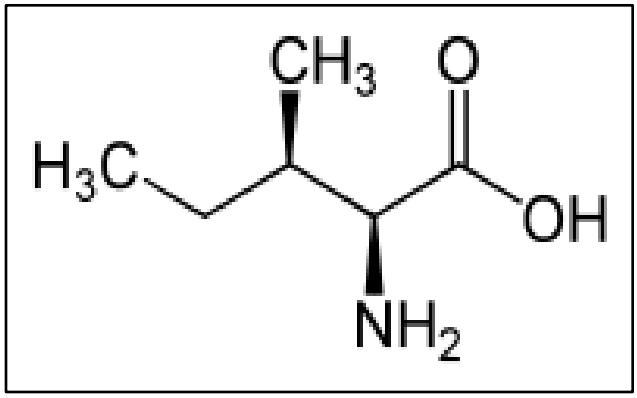
Name: Fumaric Acid



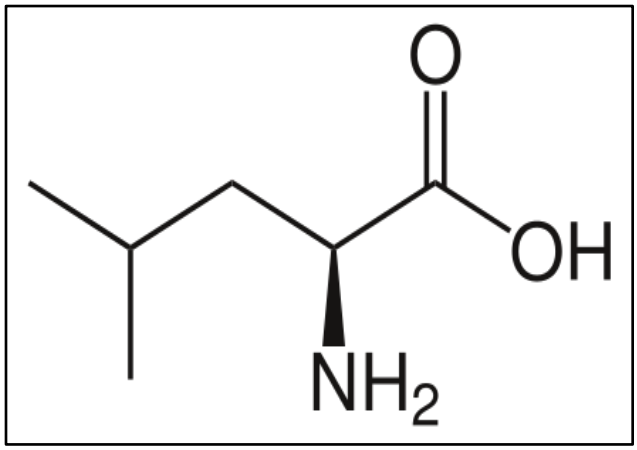
Name: Pipecolic Acid



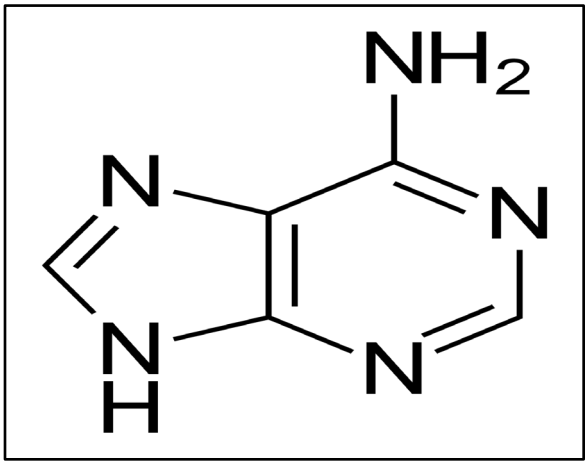
Name: Isoleucine



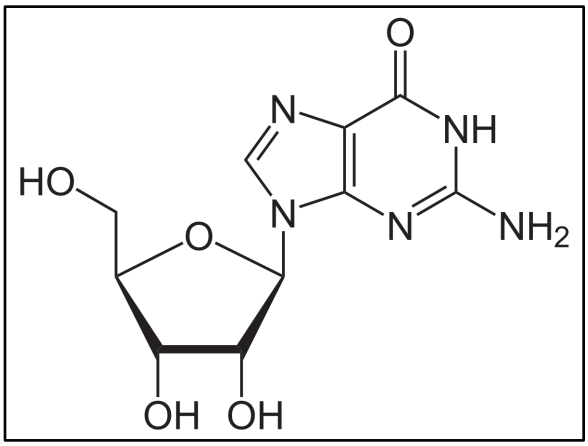
Name: Leucine



Name: Adenine



Name: Guanosine



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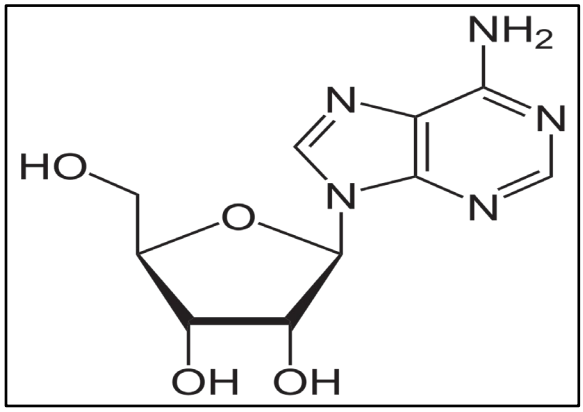

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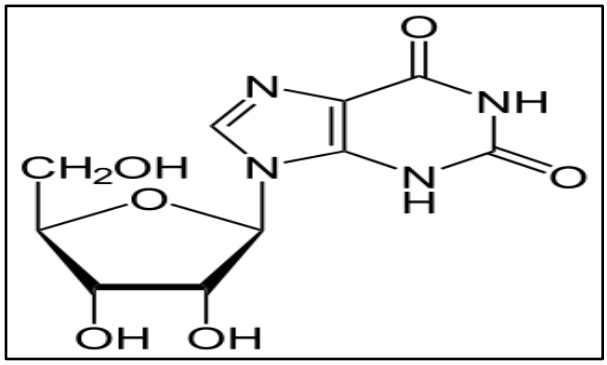
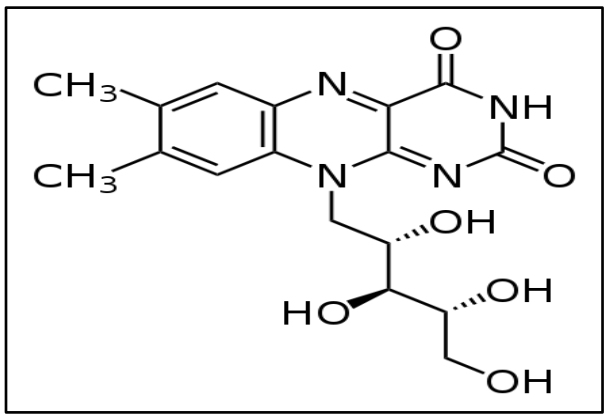
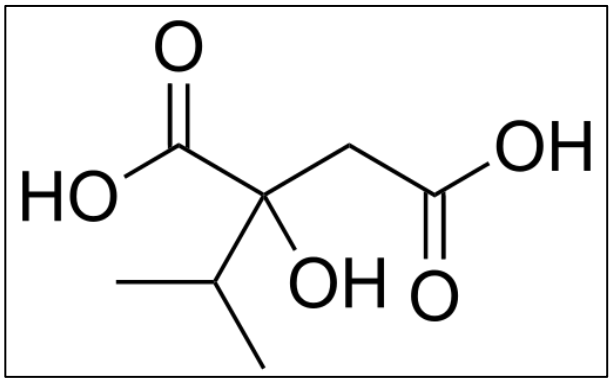


Name: Riboflavin



Name: 2-isopropylmalic Acid (internal standard)



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BIOGRAPHICAL INFORMATION

Jessica Gomez-Vargas is a first generation college student. She enjoys any opportunity that she gets to fill her brain with more knowledge. Due to her passion for the field of medicine, she plans on working hard to pursue her dream of becoming a physician. She volunteers at a children's hospital, shadows a colon and rectal surgeon, and works at a pharmacy because she enjoys her time there. She came across an opportunity to conduct research on pathogenic bacteria found in drinking water that lead to human diseases and was eager to learn more about the current studies being done pertaining to the health field.