LONG-TERM STORAGE STABILITY AND OXIDATION PRODUCTS OF THE CYANIDE ANTIDOTE DIMETHYL TRISULFIDE

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A Thesis

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Master of Science

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by

Indika K. Warnakula

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LONG-TERM STORAGE STABILITY AND OXIDATION PRODUCTS OF THE CYANIDE ANTIDOTE DIMETHYL TRISULFIDE

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DEDICATION

I would like to dedicate my thesis to:

- My greatest research supervisor, Dr. Ilona Petrikovics, whom I respect as my academic mother.
- My parents who gave me birth to this world.
- My loving and caring wife, Pamodha Madhubhashinie, who makes my life easy and comfortable.
- All the teachers who taught me and helped me to enhance my academic knowledge at Sam Houston State University.

ABSTRACT

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Exposure to cyanide (CN) leads to the malfunction of mitochondrial activity by inhibiting the terminal oxidase, cytochrome c oxidase. The brain and heart are the highest oxygen consumers and are the critical target organs harmed by CN. Dimethyl trisulfide (DMTS) is a well-studied CN antidote with the major mechanism of sulfur donation for CN conversion to SCN. However, recent investigations revealed a minor mechanism of methemoglobin formation from DMTS that is a CN scavenger type antidote. Formulation efforts of DMTS focus on optimizing pharmacokinetic and storage stability parameters. This study reports the storage stability of a formulation for DMTS (F3-formulation) developed by Southwest Research Institute, San Antonio, Texas. The F3-formulated DMTS was stored in glass ampules at 4, 22 and 37 ºC. At regular time intervals over the period of one year, nine ampules (three stored at each temperature) were opened and analyzed by HPLC-UV to determine their DMTS content. The results showed that there was no measurable loss of DMTS over the one-year period of the study for the samples stored at 4 and 22 ºC. The samples stored at 37 ºC showed good stability for five months. However, in the sixth month of storage, these samples showed a 10% (M/M) decrease in DMTS content. Discoloration and the appearance of a new peak in the HPLC chromatogram accompanied this loss of DMTS. Continued growth of these new peaks and deepening discoloration was observed over the final 5 months of the study. To identify the degradation products formed during storage at 37 ºC, separate oxidation studies were performed with DMTS using strong oxidizing agents, such as mCPBA or H_2O_2. The products of the oxidation studies were compared to those of the stability study samples. Dimethyl tetrasulfide and dimethyl pentasulfide were observed as products of both studies. Dimethyl disulfide was observed as a product of degradation and *S*-methyl methanethiosulfonate was revealed as a product of oxidation. The HPLC and GC-MS SPME analysis revealed a good agreement between the degradation products of the stability study and the direct oxidation reactions. Because the 22 and 4 ºC samples remained stable for a year, we can conclude that the F3-formulated DMTS fulfills the criteria for storage stability.

KEY WORDS: Cyanide, Antidote Formulations, DMTS, Long-term Storage Stability, Ampule Sealing, HPLC, GC-MS, Degradation, Oxidation products.

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CHAPTER I

INTRODUCTION

Sources and Toxicity of Cyanide

Cyanide Sources

The word "cyanide" (CN) originated from the Greek word *kyanos* meaning dark blue. CN can be found everywhere in the cosmos and in various spheres of nature. CN sources can be natural or man made. The natural CN sources are cassava root, yams, maize, bitter almonds, apricot, apple and peach seeds, where CN is present as cyanoglucosides.[1] Other than that, lightning and volcanic eruption can easily form CN in the environment.[2] Some fungi, bacteria and animals produce CN for self-defense purposes. CN is present in smoke from: cigarette, and fires (fueled by nitrogen containing molecules, acrylonitrile, wool, silk).[3] CN is used in the electroplating industry,[4] gold and silver extraction industry,[5] and in the battery the industry.[6] Hydrogen cyanide, the acidic form of CN, was first isolated from cherry laurel by a Swedish chemist, Carl Wilhelm Scheele, in 1782.[7] Since it appears as white crystals, many have misidentified it as white sugar or salt.[8] The basic form of CN is the cyanide anion, CN^-. It has a molecular weight of 26.018 g/mol and has a formal a charge of -1 which is a supportive for complexation.[9] In ancient Egypt, CN was used as one of the toxic compounds for judicial executions (they have identified high content of CN in peach seeds),[10] and also as an additive of a gold containing drug developed in ancient Chinese experiments.[11] Very recently CN was used as a warfare agent: HCN gas was used in World War II by the Nazis against the innocent holocaust[12] victims and by the Liberation

Tigers of Tamil Eelam (LTTE) terrorist group as a suicidal agent to be taken by members who were captured by the Sri Lankan government army.[13]

Toxicity of CN

CN is a very toxic agent, which inhibits the cellular oxygen utilization of living beings. The possible toxicity may occur through inhalation of smoke from industrial or residential activities, oral administration through food and drinks and dermal absorption threat attack.[14,15] Exposure to CN through one of these routes may cause severe illness or death. CN enters into the mitochondria of the living cell and binds to the terminal enzyme of the electron transport chain cytochrome c oxidase, resulting in acute cellular hypoxia, which prevents the production of ATP inside the cell.[16] Due to these actions, the anaerobic pathway becomes dominant, and promotes the reduction of pyruvate to lactic acid resulting in lactic acidosis. This leads to central nervous system and myocardial depression.[3] CN can be present in the molecular form of hydrogen cyanide (HCN) and in the anion CN^-. The ratio of HCN:CN^- forms of CN in the human body is approximately 63.1. The pKa of HCN is 9.2. HCN can easily penetrate through the cellular and subcellular membranes.[3] High doses of CN (~LD_{50}) are able to produce more critical symptoms such as induction of pulmonary arteriolar and coronary vasoconstriction, which can cause pulmonary edema or cardiogenic shock. The lower doses of CN result in dizziness, headache, vomiting and nausea due to the inhibition and interference with cellular enzymes.

Antidotes for CN Intoxication

Different Types of CN Antidotes

Several types of CN antidotes were identified after many years of research. The two identified mechanisms of action to treat CN intoxication are the use of scavengers and detoxification agents.

Scavengers

This type of antidote can form stable complexes with CN, such as methemoglobin or cobalt compounds. Amyl nitrite, sodium nitrite and 4-dimethylaminophenol are examples of methemoglobin formers (Figure 1).

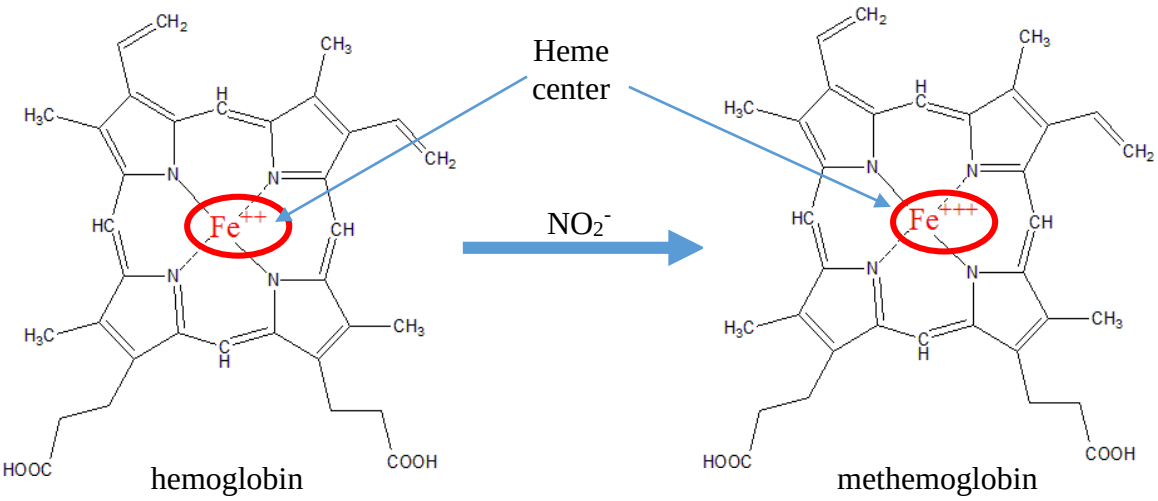


Figure 1. Conversion of Hemoglobin to Methemoglobin by Nitrite Anion.

In the US, the currently available major CN antidotes are the *Nithiodote*™ and the *Cyanokit*®. *Nithiodote*™, is a scavenger type antidote that contains a combination of sodium nitrite and sodium thiosulfate (TS). Nitrous acid has a pKa of 3.15 and present in dissociated form of nitrite at body pH. Sodium nitrite can convert oxyhemoglobin to

methemoglobin by changing the oxidation state of the heme center from Fe^{2+} to Fe^{3+} (Figure 2). Methemoglobin has the ability to trap the CN^- anion in its heme center and to form cyanomethemoglobin. Rhodanese (Rh) converts CN to the less toxic thiocyanate (Figure 3) with TS and is excreted form the body with urine.

$$Fe^{2+}_{(aq)} + NO^-_{2\,(aq)} + 2H^+_{(aq)} \longrightarrow Fe^{3+}_{(aq)} + H_2O_{(l)} + NO_{(g)}$$

Figure 2. Oxidation State Conversion of the Iron Center by Nitrite.

$$S_2O^{2-}_{3\,(aq)} + CN^-_{(aq)} \xrightarrow{\text{Rh}} SCN^-_{(aq)} + SO^{2-}_{3\,(aq)}$$

Figure 3. Conversion of the Toxic CN into the Less Toxic SCN in the Presence of Rh.

Cyanokit® contains hydroxocobalamin as the active agent. Hydroxocobalamin can bind CN^- to its Co^{2+} containing metal center and convert it to cyanocobalamin (Figure 4). With this process, the body easily removes CN and reduces its toxic effect.

Both of these antidotes have the limitation that they require intravenous (IV) administration, which is impractical when treating elevated number of victims. In addition, TS has low sulfur donor efficacy, which is unfavorable to CN conversion to SCN^-. The presence of endogenous Rh enzyme catalyzes the conversion of CN to SCN and TS. Excessive use of nitrite may lead to the production of excess methemoglobin, which causes methemoglobinemia resulting in the reduction of oxygen transportation. *Cyanokit*® needs *in situ* preparation and high injection volumes (>200 mL).[17] Due to the identified drawbacks, scientists studied the sulfur containing molecules which can donate "S" easily. The journey of investigating new CN antidotes resulted in three new types of antidotes: a) sulfur donor molecules such as dimethyl trisulfide (DMTS), b) cobinamide (successor of

hydroxocobalamin), and c) sulfanegen (successor of the mercaptopyruvate).[18,19] Figure 5 shows the mechanism of sulfur donation.

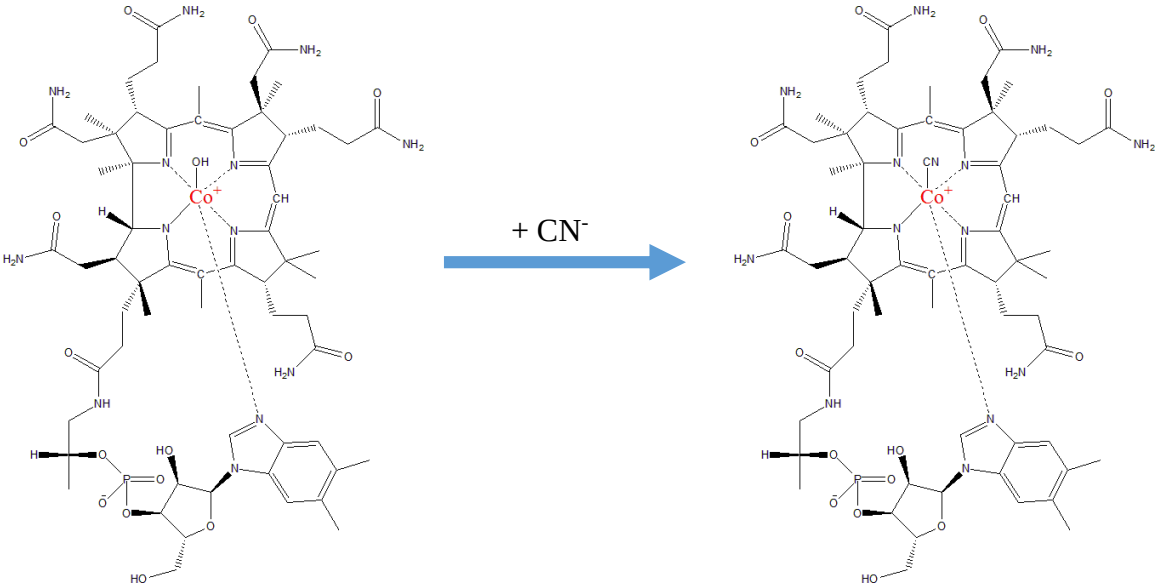


Figure 4. Hydroxocobalamin Conversion to Cyanocobalamin in the Presence of CN.

$$CN^-_{(aq)} + S\ (sulfur\ donor) \longrightarrow SCN^-_{(aq)}$$

Figure 5. CN Conversion to SCN in the Presence of a Sulfur Donor.

Detoxifiers

Detoxifiers are molecules that convert CN into the less toxic SCN or into a different component, which is easily excreted from the body. The recently understood detoxifiers are TS, sulfanegen, 3-mercaptopyruate (3-MP), cystine and α-ketoglutarate (α-KG).[20–22] Rh and 3-mercaptopyruate sulfurtransferase enzymes catalyze the CN conversion to SCN in the presence of a sulfur donor. Here both TS and 3-MP act as sulfur donors. Cystine acts as another detoxifier, which removes CN from the medium by producing 2-amino-2-thiazoline-4-carboxylic acid (ATCA). Other than the previously discussed detoxification

mechanisms, there is an alternative detoxification process, in which CN reacts with α-KG. Here the formation of α-ketoglutarate cyanohydrin eliminates CN. α-KG is found in all aerobic organisms. Reactions of CN with detoxifiers have been summarized below (Figure 6).[21]

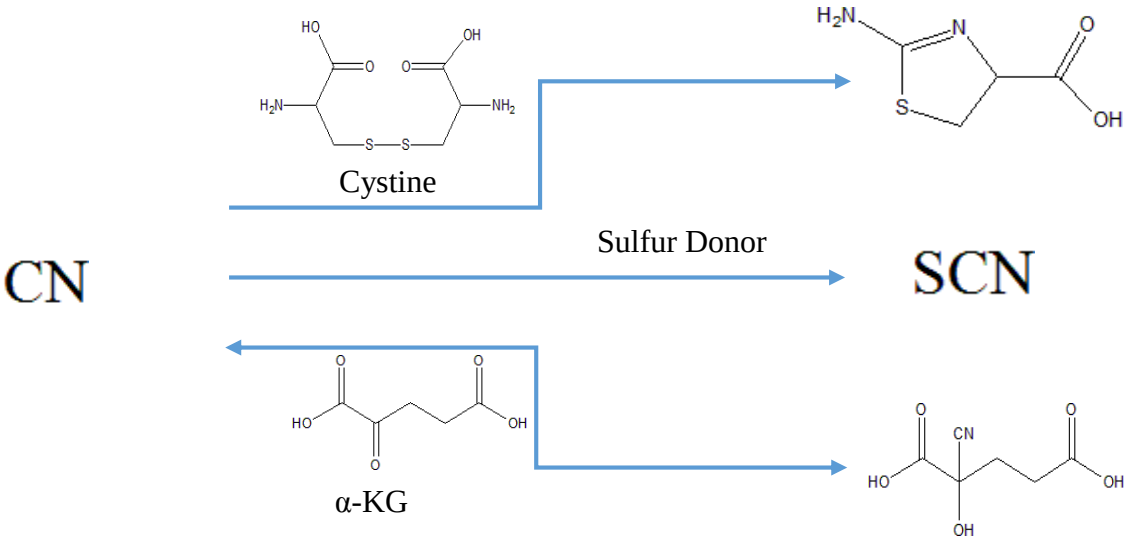


Figure 6. CN Detoxification Pathways. *Based on Mitchell et al.*[21]

Another recently discovered detoxifier molecule is DMTS which acts as a sulfur donor. This molecule also has the ability to convert hemoglobin into methemoglobin, which is considered as a scavenger.[17,23]

Dimethyl Trisulfide (DMTS)

DMTS is used as a flavor enhancer and food additive in the food industry. It is produced in fungating cancer wounds.[24] DMTS is also present in many natural sources such as broccoli, cabbage, cauliflower, onion and in the highest concentration in garlic. In this plant a series of lipophilic molecules are produced by the decomposition of alliin

catalyzed by the alliinase enzyme. One of these products is allicin, which can be extracted with ethanol and gives the characteristic garlic odor. The majority of decomposition products are soluble in fats, oils and non-polar solvents, but insoluble in water which is polar.[17] Alliin also undergoes different reactions to produce various di- and polysulfide compounds. Sulfur containing compounds in garlic, such as DMTS and diallyl disulfides, have been studied as sulfur donating compounds which can be used as CN antidotes (Figure 7).[25] Several sulfur donor type molecules have been tested and the garlic component DMTS proved to be the best CN antidote. The application of DMTS and its formulation as a CN antidotes patented by Dr. Petrikovics's lab (Sam Houston State University, Huntsville, Texas) under the title of CN antidote compositions (US 20150290143 A1, 2015; US 20150297535 A1, 2015).[26,27] Unlike TS, DMTS can convert CN to SCN with high efficiency with and without Rh, and has a higher antidotal potential than TS in the same dose. The intramuscular (IM) administration makes formulated DMTS superior over *Nithiodote*™ and *Cyanokit*® as victims can self-administer the antidote during CN exposure.

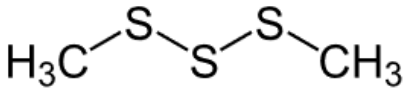


Figure 7. Structure of DMTS.[28]

Formulated DMTS

Literature data showed that DMTS has higher efficiency compared to TS to donate a sulfur atom to CN.[23] The middle sulfur atom of DMTS is the one proposed to, which binds to CN. The next step was to develop formulations for IM administration and to characterize the formulations *in vitro* and *in vivo.* Micelles can provide a suitable carrier

system for the lipophilic DMTS to penetrate the blood brain barrier with the help of their hydrophilic corona although their core is hydrophobic.[15] The goal of the development of formulations for DMTS is to achieve a set of characteristics such as suitability for IM, increased solubility, improved stability *in vivo* and *in vitro*, improved *in vivo* antidotal efficacy, enhanced bioavailability and improved absorption kinetics. Development of formulations can be achieved using three different criteria such as solvent combinations, co-solvents and surfactants. One of the DMTS formulations developed by Dr. Petrikovics's lab is 15% (m/v) polyoxyethylenesorbitan monooleate 80 (poly 80). There are three different types of DMTS-related formulations used in Dr. Petrikovics's lab. These formulations are labelled as F1, F2 and F3 according to their used chronological order.

- F1-Formulaton : Lipid-Based Formulation (micellar or liposomal encapsulation)
- F2-Formulation : 15% (m/v) poly 80 in ethanol (EtOH) – (patented at Sam Houston State University, Huntsville, Texas)
- F3-Formulation : poly 80 : sorbitan monooleate (span 80) (3:1) – (developed at Southwest Research Institute - SwRI, San Antonio, Texas)

After developing an efficient DMTS formulation, it needed to be characterized for future use. Stability, size distribution, *in vivo* efficacy, pharmacokinetics, blood-brain barrier penetration and organ distribution experiments are examples for some of the characterization experiments. The major focus of this thesis project was the investigation of storage stability.

Lipid-Based Formulated DMTS

One of the earliest approaches was the development of the lipid-based formulation. This formulation was developed based on using of co-polymer PEG2000-DSPE. The preparation of the formulation had five consecutive steps, Step 1: preparation of PEG2000-DSPE stock solution in EtOH. Step 2: formation of the lipid film. Step 3: rehydration of the lipid film to target concentration. Step 4: addition of DMTS. Step 5: vortexing at 50 ºC for 20 minutes. The concentration of DMTS in this formulation was found to be 2.5 mg/mL. Instability and low encapsulation efficiency of this lipid-based formulation led to further investigations on new formulations.[15,29]

15% Poly 80 Formulated DMTS

This formulation was prepared by mixing pure DMTS with 15% (m/v) aqueous poly 80 solution. The solubility of DMTS in water is only 0.13 mg/mL,[25,30] therefore it was necessary to enhance this feature. The surfactant poly 80 helped to dissolve DMTS in water up to 86 mg/mL concentration.[30,31] Former member of Dr. Petrikovics's research group Dr. Lorand Kiss compared the storage stability of the 15% (m/v) poly 80 formulated DMTS in snap-cap and crimp-sealed vials versus ampules. DMTS in EtOH was stable for few hours. Reduction of 36%-58% (M/M) in DMTS content in 15% (m/v) poly 80 formulation stored in snap-cap and crimp-sealed vials within a 29-week period was previously reported.[25]

Because of the low stability in snap-cap and crimp-sealed vials, hermetically fire-sealed ampules were used for the storage of the formulated DMTS. The study has reported that the F2-formulated DMTS in hermetically fire-sealed vials was stable for 100 days. This suggested the suitability of hermetically fire-sealed vials for the storage of F3-formulated DMTS.[30] The maximum concentration of DMTS in the F2-formulation was

reported as ~86 mg/mL.[32] The identified disadvantage of this formulation is its impracticality for applications in mass scenarios, since it needs high injection volumes. Therefore, the development of more suitable formulations with higher antidote concentrations were conducted.

Poly 80 : Span 80 (3:1) Formulated DMTS

This formulation was developed in Southwest Research Institute, San Antonio, Texas. The initial goal of this new formula is to overcome the low stability of F1 and the high injection volume requirement of F2. This formulation was prepared using poly 80 and span 80 in a ratio of 3:1 (*m/m*). The ratio of DMTS is 40% (*m/m*) in the formulation. Therefore, the requirement for a low injection volume can be fulfilled with this formulation. The F3-formulation still needs to be characterized before future use. Recent studies found its slower absorption rate as one of the disadvantages. There were several studies carried out for the characterization of the F3-formulation, their results will be published in the near future.

Redox Reactions with DMTS

It was reported that dialkyl polysufides undergo disproportionation reactions.[33] The reaction rate of disproportionation depends on the temperature, it is specially faster at high temperatures such as above 145 – 160 ºC.[33] The thermally decomposed products are also unstable and they undergo further decomposition. According to the results, disproportionation of DMTS could produce dimethyl sulfide (DMS), dimethyl disulfide (DMDS), dimethyl tetrasulfide (DM4S) and dimethyl pentasulfide (DM5S). There is a possibility of dimethyl hexasulfide to occur also, but the higher the number of sulfur atoms

in the sulfur chain the lower the bonding energy. Primarily, symmetrical cleavage of the sulfur chain was observed in the majority of the cases, while asymmetrical cleavage was secondary. Generation of elemental sulfur was also observed during those studies.[33]

Through reduction reactions, thiols and hydrogen sulfide were formed, while the release of sulfur atom radicals from polysulfides were reported.[34,35] When oxidation reactions took place, thiosulfate and thiosulfonate were formed from the organic polysulfides.[36] Certain studies reported the production of dimethyl thiosulfinate also (Figure 8). The DMTS molecule was oxidized by 30% hydrogen peroxide (H_2O_2) in the presence of HNO_3. Some of the literature have brought up oxidation of all three sulfur atoms, two sulfur atom and one sulfur atom in DMTS, and oxidation of terminal sulfur atom. It is possible that along with the disproportionation, an oxidation reaction occurred due to the small amount of air within the hermetically fire-sealed glass ampules.[16,37,38]

According to the literature, the following reactions could occur with DMTS (Figure 8).

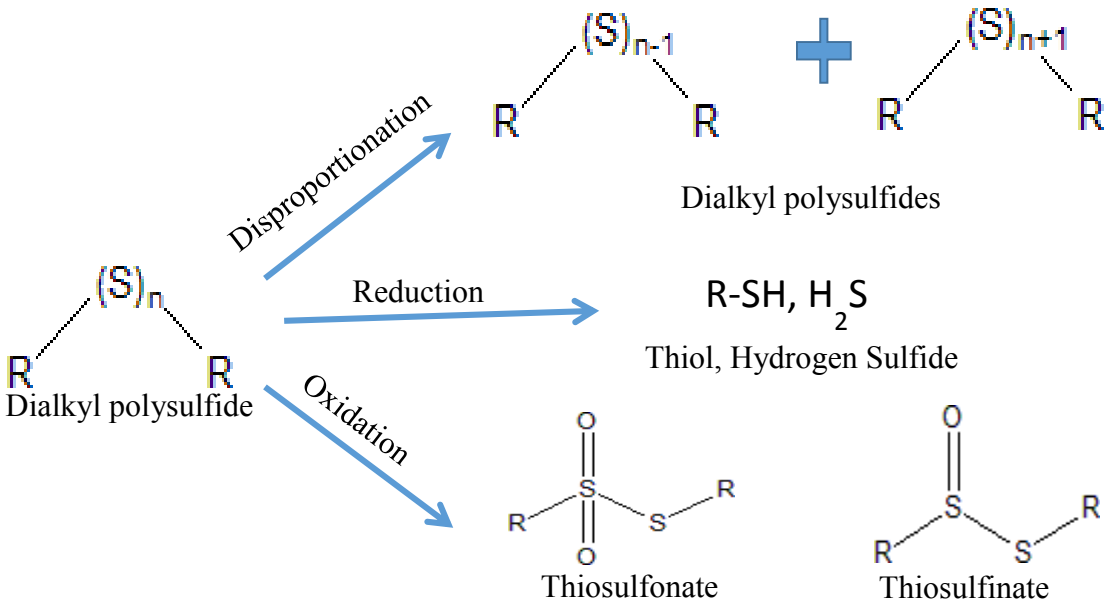


Figure 8. Possible Reaction Products of Dimethyl Polysulfides.

Analytical Techniques

Gas Chromatography - Mass Spectrometry (GC-MS)

GC-MS is a combined instrumental technique, which allows separation, quantification and identification of components of complex solution mixtures. This is one of the well-known and common methods for identification of molecules of complex mixture. GC can separate molecules from each other based on their polarity and affinity to the column stationary phase. For example, if the stationary phase is non-polar, the non-polar components will be retaining longer and the polar components elute with the mobile phase sooner. The ion source in the MS breaks the molecules into fragments by ionizing them and later they can be separated depending on their mass-to-charge ratios (m/z). Based on the amount of similar molecules or fragments of the components, the area under the curve for quantification purposes can be determined. The important components of GC-MS will be explained below (Figure 9).[39]

The high purity gas source is one of the important requirements. Usually these gases are prepurified, although the instrument passes them through a filter system for safety and quality reasons. It is important to maintain the required pressure and flow of the gas for analysis. Therefore, the gas supply is controlled with a pressure regulator. Several different types of gases are used in GC depending on the detector: the carrier gas can be either H_2, He or N_2.[40,41]

There are sets of pneumatic controls in the GC depending on the type of analysis and the type of detector. GC uses a split/splitless inlet and it requires the maintenance of the carrier gas supply pressure, column inlet pressure, inlet septum purge flow and inlet split flow. Basically, all the operations of the GC-MS instrument are maintained through

pneumatic controls. Most of the modern instruments are coupled with a PC for electronic control and for ease of use, although older instruments may have manual pressure control via regulators.[40,42]

The samples usually inject to the column through inlet. Usually, the temperature in the inlet is higher than in the oven. The sample can easily evaporate and enter to the carrier gas (mobile phase). In the lab we use split/splitless inlet.

The retention of analytes depends on the interactions with the column. This is similar to HPLC or TLC. Interactions between the stationary phase and compound are important for analysis. There are three bonding categories depending on interaction type: hydrogen bonding, dipole and dispersive. The analyte mixtures are separated into groups on the column. The columns divide into two main types: they can be capillary or packed. That varies with application type and the affects length and internal diameter. Capillary columns are long hollow silica pipes. Their wall is covered with the stationary phase on the inside. Many different stationary phases are available for different applications.

The oven contains the GC column. This oven can be heated or cooled. This difference in temperature helps to separate compounds. The sample enters onto the column from the inlet through the injector connection and exits through MS interface. We use a temperature program during the analysis to separate the products of the stability study and the oxidation reaction.[40]

The detector responds to a physicochemical property of the analyte. After amplification, an electronic signal is generated. Based on that the computer draws the chromatogram. The software can use this information for qualitative and quantitative

analysis also. One can select the detector type based on application and sensitivity. In the lab, we use the mass spectrometer as the detector.[39,40]

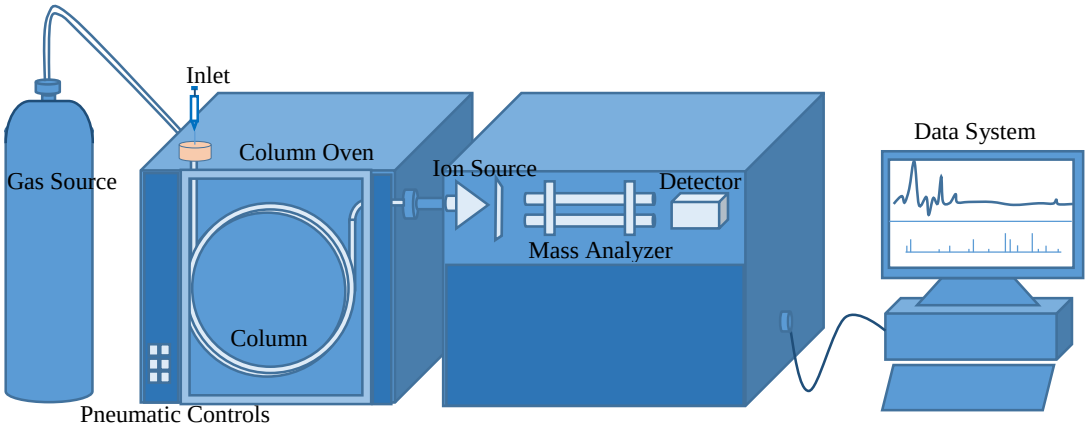


Figure 9. Schematic Diagram of a GC-MS Instrument.

High Performance Liquid Chromatography (HPLC)

HPLC is an improved form of liquid chromatography. The mobile phase is forced through the column at pressures as high as 420 atmospheres. This is a fast process compared to gravitational liquid chromatography. All chromatographic separations are based on the same principle. Separation of the components of the mixture is based on their affinity to stationary phase and the mobile phase. There are several types of HPLC, which are listed below.[39,43,44] Normal phase-, reverse phase-, adsorption-, ion-, and size exclusion chromatography are the examples for the type of HPLC. We used a reverse phased column (C8) during our experiments. As shown in the schematic diagram (Figure 10) the HPLC instrumentation includes major components of solvent reservoir, high pressure pump, sample injector, column, detector and data acquisition and display system. The heart of the system is the column where the separation occurs.

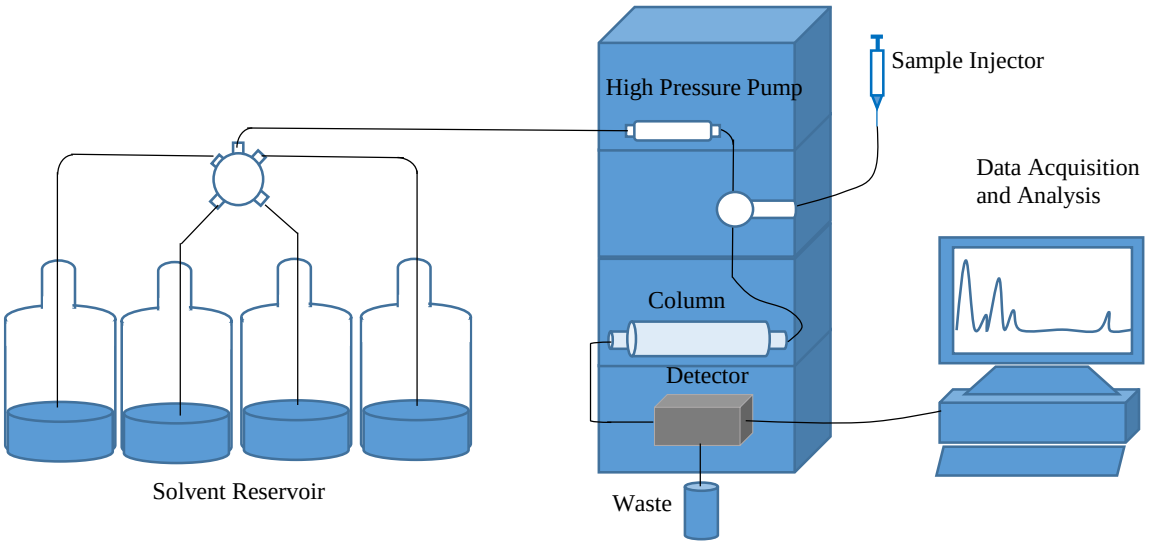


Figure 10. Schematic Diagram of the HPLC Instrument.

The mobile phases are placed in glass bottles. They are usually a mixture of polar and non-polar liquid components. The pump forces the mobile phase from the solvent bottles through the column and the detector. The flow rate depends on the dimensions of the columns, the stationary phase particle size and the composition of the mobile phase.

The injector can be manual or automated. It should provide injection of the liquid sample within the range of volume 0.1-100 µL. Columns are made of stainless steel with an internal diameter of between 2 and 20 mm. They are filled with a stationary phase where the particle size is between 3–10 µm. Unlike in GC-MS the temperature of the HPLC column is kept constant. The detector reveals the analytes in the order of elution. Our HPLC is equipped with UV/Vis and fluorescence detectors. Signals from the detector are collected and sent to electronic integrators. The connected computer processes the chromatographic data.

Thin Layer Chromatography (TLC)

In the TLC, a solid phase is coated onto a solid support as a thin layer. During the experiments, we used aluminum sheets as solid support. The mixture of components is dissolved and the solution is spotted near the bottom of the thin layer plate. They are left to dry for a few minutes. Based on the capillary effect, the eluent is flowing from the bottom to the top. If the eluent is non-polar the non-polar components in the mixture travel faster. However, the eluent is polar, the polar components of the mixture will travel faster according to the normal phase stationary phase. After drying the plate we used UV-light to visualize the spots (Figure 11).[45–47]

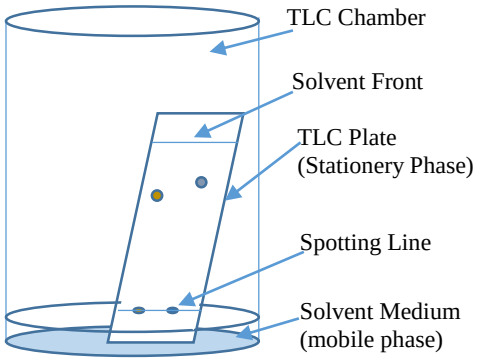


Figure 11. Setup of the Analysis on TLC Plate.

CHAPTER II

MATERIALS AND METHODS

Chemicals

All the chemicals used were commercially available. DMTS, DMDS, calcium carbonate, calcium bicarbonate, hexane (C_6H_{14}), ethyl acetate (EtOAc), H_2O_2, acetone and EtOH were purchased from Sigma-Aldrich (Milwaukee, WI, USA), *S*-methyl methanethiosulfonate (SMMTS) was purchased from Sigma-Aldrich (St. Louis, MO, USA), meta-chloroperbenzoic acid (mCPBA), acetonitrile and water (HPLC-grade) was purchased from Acros Organics (Thermo Fisher Scientific, Waltham, MA USA). Poly 80 was from Alfa Aesar (Tewksbury, MA, USA), and Span 80 was purchased from TCI (Toshima, Kita-Ku, Tokyo, Japan).

Instruments

Table 1 lists the instruments used in these experiments.

Table 1

Analytical Instruments Used in this Research.

Instrument	Brand and Model	Location
HPLC	Thermo Scientific Dionex Ultimate 3000	1
GC-MS	Agilent Technologies 7890A GC System and 5975C VL MSD	1
Ampoule Sealer	Unbranded RF-1 Ampoule bottle sealing machine 201698	1
Analytical Scale	Mettler Toledo New Classic MF ML204/03	1
pH meter	Thermo Scientific Orion Star A111	1
Centrifuge	VWR International Galaxy 20R	1
Auto Vortex	Heidolph Multi Reax	1
TLC Plate	Agela Technologies, T-CSF200200-A	1
SPME Holder	Supelco Analytical, 57330-U Manual	1
SPME Fiber	Agilent Technologies SPME Fiber Assembly 391896301	1
UV-light	UVP LLC, UL Listed Insp. & Meas. EQ 399-J UVGL-25	2

*1 – Dr Petrikovics's Lab 2 – Dr. Gross's Lab

Methods

PART 1

Preparation of the F3-Formulated DMTS Solution for the Long-Term Storage Stability Study

This formulation was prepared according to the protocol provided by the Southwest Research Institute (SwRI), San Antonio, Texas. It contains poly 80 and span 80, without any water. An amount of 99.57 g of poly 80 and 33.20 g of span 80 were weighed into a 10 ml glass bottle and hand vortexed for 5 minutes, then DMTS (88.5 g) was added to prepare 221 g of stock solution. The resulting mixture was hand-vortexed (VWR Fixed Speed Mini Vortex) for further 5 minutes, followed by auto-vortexing (2000 RPM speed) (Heidolph Multi Relax Auto-vortex) for 30 minutes. This gave a clear, yellowish solution.

Storage of F3-formulated DMTS

The prepared F3-formulated DMTS was transferred into glass ampules. A total number of 189 glass ampules were used. Each glass ampule (2.0 mL) was filled with 1 mL of F3-formulated DMTS and sealed using an ampule sealer (Figure 12). Before fire-sealing, each glass ampule was examined to ensure it did not have formulated DMTS on the wall of the neck of the ampule.

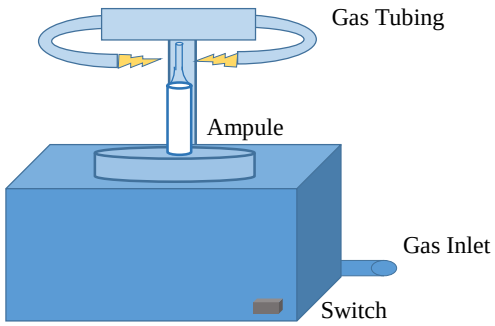


Figure 12. The Ampule Sealer.

The sealed glass ampules were separated into 3 groups and were color coded with blue (4 ºC), yellow (22 ºC) and red (37 ºC) tape. When storing the fire-sealed glass ampules the following scheme was applied (Figure 13).

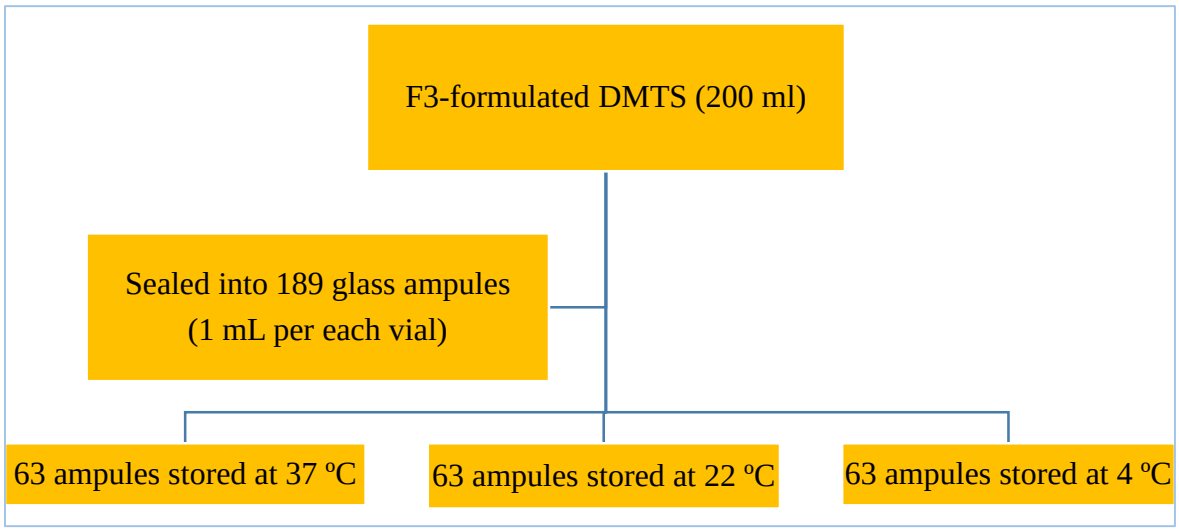


Figure 13. Stability Study: Glass Ampule Storage Criteria.

The glass ampules were examined for proper sealing and defective fire-sealed ampules were removed from the quota. The grouped fire-sealed glass ampules were transferred to plastic ampule holders and stored in a fridge, in a cabinet, or in an oven (Figure 14). The temperatures were monitored at each sampling point to keep track of the temperature fluctuations.

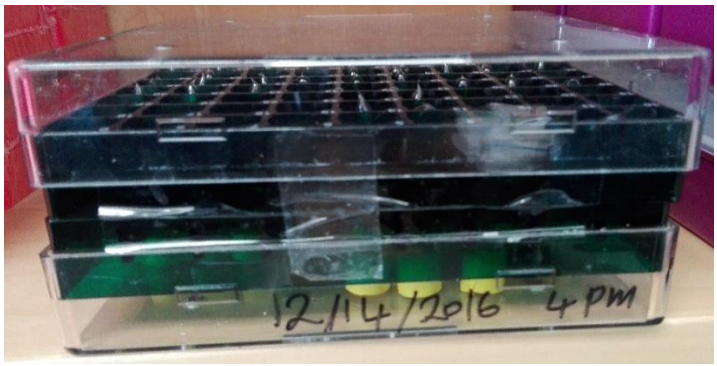


Figure 14. Room Temperature (22 °C) Stored, Ampule-Sealed, F3-formulated DMTS.

The stored DMTS containing glass ampules were analyzed according to the sampling point of 0 to 6 days, 1 to 3 weeks and 1 to 12 months.

Analytical Method Development for the Measurement of DMTS content in the F3-formulated DMTS by HPLC-UV/Vis

A method to analyze DMTS in blood by HPLC-UV was published earlier by Dr. Petrikovics's lab.[19] This method was modified to analyze the low DMTS concentration levels in aqueous medium of the parallel artificial membrane permeability assay samples.

Preparation of a calibration curve for the analysis of the F3-formulated DMTS

F3-formulated DMTS (400 mg/mL DMTS) was diluted in two steps with EtOH to obtain a working solution of 0.05 mg/mL DMTS. All the solutions were prepared in glass vials and micropipettes with plastic tips were used for solutions transfers. The stock F3-formulated DMTS (50 µL) was diluted with EtOH (4950 µL) to obtain 4 mg/mL solution. First diluted solution (62.5 µL) was diluted with EtOH (4937.5 µL) to obtain 0.05 mg/mL solution. Using direct dilution with 0.05 mg/mL F3-formulated DMTS the standard solutions of blank, 0.01, 0.02, 0.03, 0.04 mg/mL were prepared. The HPLC analysis was initiated by pipetting 600 µL solution out of the double diluted F3-formulated DMTS in EtOH to an HPLC vial. Then an aliquot of 400 µL of 0.1 mg/mL DMDS in acetonitrile was added to the vial. DMDS was used as the internal standard to avoid injection errors. The vial (Figure 17) was closed tightly with a screw-type cap, auto-vortexed for 10 seconds and mounted in the auto-sampler of the Dionex Ultimate 3000 (Thermo Scientific, Waltham, MA, USA) HPLC-UV instrument in Dr. Petrikovics's lab. From the 1 mL sample mixture 40 µL was injected to the guard column (product number: KJ0-4282,

Phenomenex) connected to the 250 x 4.60 mm non-polar C-8 analytical column (product number: 00G-4250-EO, Phenomenex Luna, pore size of 100 Å, outer diameter 5 µm). The mobile phase consisting of 35% (v/v) water and 65% (v/v) acetonitrile was used with a 1 mL/ min flow rate in isocratic mode. The absorbance of the eluate was monitored at 215 nm by a UV detector. After the sequence run was completed, the chromatographic peaks were analyzed by the HPLC instrument software (Thermo Scientific Dionex Chromeleon 7 version 7.2.0.3765). The peak area ratio of DMTS and DMDS was plotted against the DMTS concentration to obtain the calibration curve.

Analysis of F3-formulated DMTS at each sampling point

The internal standard of 0.1 mg/mL DMDS in ACN was freshly prepared from pure 10 mg/mL DMDS (50 µL) diluted in ACN (4950 µL) solution. The stock 10 mg/mL DMDS solution was prepared by mixing 50 mg of pure DMDS in 5 mL of EtOH. Then three ampules from each temperature (Figure 15) were taken and left until they reach at room temperature. It was necessary to identify the proper dilution ratio for the analysis of the stored samples of the F3-formulated DMTS in HPLC-UV/Vis. First 50 µL aliquots from ampules were pipetted out and diluted with 4950 µL of EtOH (Figure 16a). From the first diluted sample, 50 µL were pipetted out and diluted with 4950 µL of EtOH to prepare the second diluted sample (Figure 16b). At the next step, diluted samples from the first and second dilution were mixed respectively with 600 µL and 400 µL of 0.1 mg/mL DMDS internal standard solution. The samples were analyzed at every sampling point during the study period. Samples were analyzed by the same instrumental method that was used when preparing the calibration curve. Since the single dilution showed a significantly higher peak area for DMTS and DMDS, the double dilution method was used for the later analysis.

Similarly, peak area ratios were used for the data analysis and concentrations were determined using the best fit regression equation obtained from the calibration curve.

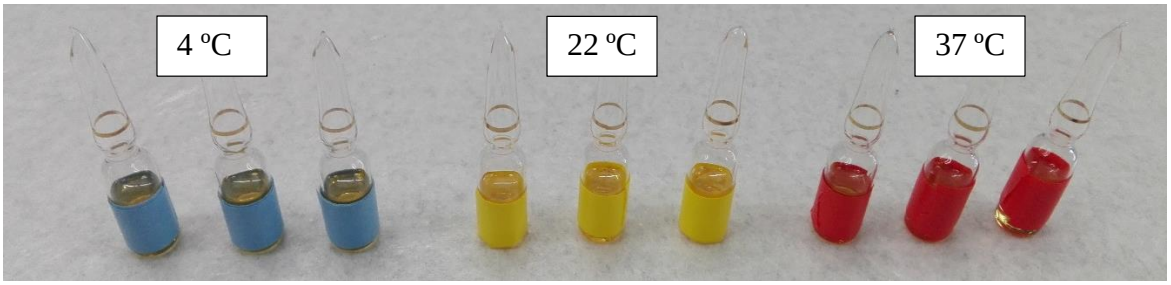


Figure 15. F3-formulated DMTS at Different Temperatures (4, 22 and 37 ºC).

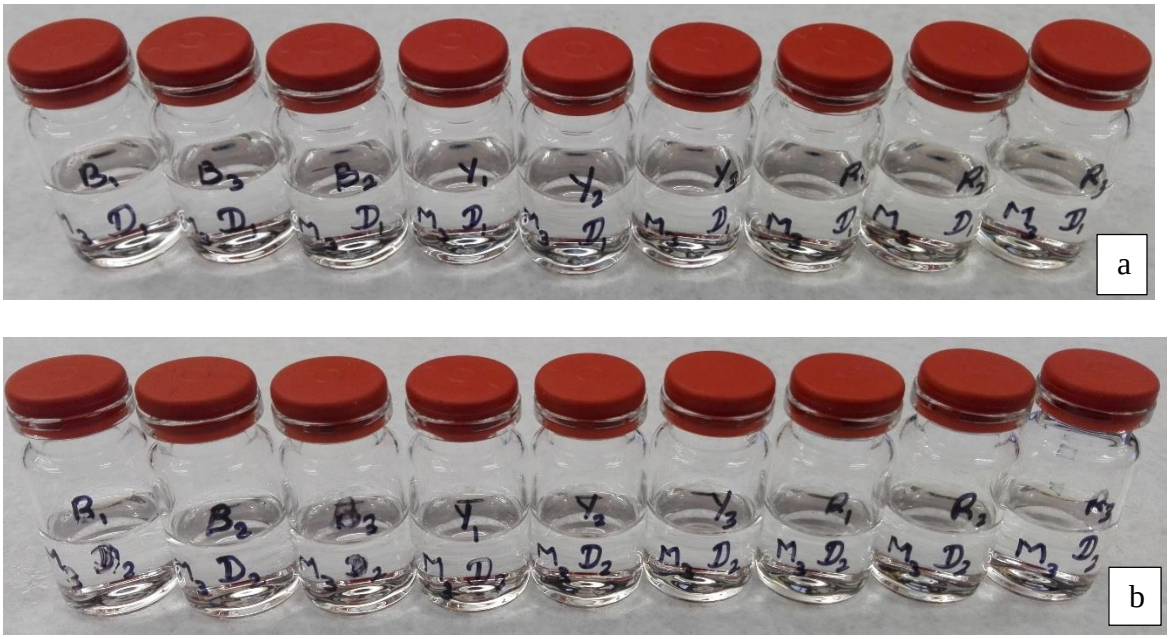


Figure 16. a) First Dilution of the F3-formulated DMTS. b) Second Dilution of the F3-formulated DMTS.

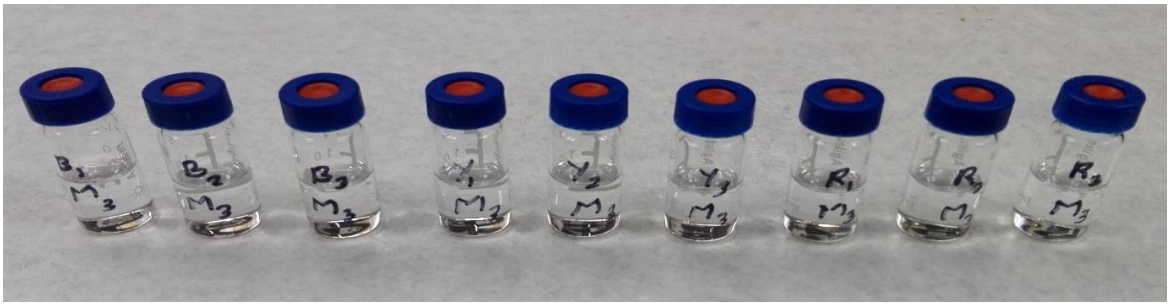


Figure 17. Instrument Friendly Samples with the Internal Standard of 0.1 mg/mL of DMDS in Acetonitrile (ACN) added.

Analysis of Long-Term Storage Stability Samples in GC-MS

Since, the samples stored at 37 ºC showed a degradation, they were analyzed using GC-MS method. The F3-formulated DMTS was high concentrated, therefore solid phase microextraction (SPME) method was used. The F3-formulated DMTS of 37 ºC stored samples from long-term storage stability study were transferred to vials directly. The sample was exposed to SPME fiber assembly (100 µm polydimethylsiloxane fused silica/SS) for 30 seconds. It was analyzed in GC-MS with inlet temperature of 180 ºC. The oven temperature was changed to 50 ºC to 280 ºC with a ramp of 40 ºC/min. The MS spectra was analyzed using MSD ChemStation Data Analysis Application. The same scenario was followed in triplicate.

PART 2

Preparation of Reactants for DMTS Oxidation with mCPBA

As reported previously[48], required amounts of DMTS and mCPBA were prepared in 99% (v/v) CH_2Cl_2. Several alterations were added to the protocol to meet the final requirements and the optimized method is described below.

Preparation of carbonate buffer solution with a pH of 9.9

Concentration of 0.100 M carbonate buffer solution was prepared mixing 0.530 g of sodium carbonate and 0.420 g of sodium bicarbonate in 50 mL of HPLC grade water. The final pH was measured using pH meter.

Preparation of DMTS solution

DMTS (2.016 g) was dissolved in 8 mL of CH_2Cl_2 and kept between temperatures of -5° and 0° C.

Preparation of mCPBA solution

mCPBA (3.040 g) was dissolved in 32 mL of CH_2Cl_2 and kept between temperatures of -5° and 0° C.

Preparation of mobile phase for TLC plate analysis

C_6H_{14} (2.1 mL of 99.5+% (v/v)) was mixed with 900 µL of 99.5+% (v/v) EtOAc and placed in a sealed glass vial.

C_6H_{14} (3 mL of 99.5+% (v/v)) was placed in a sealed glass vial.

Preparation of TLC plates for the sample analysis

The TLC plates (200mm x 200mm pH=5 MF254 aluminum back plate) were prepared with 6.5 cm length, 2.5 cm width and 2 limits were marked on the plate for starting solvent front (1 cm from bottom) and ending solvent front (0.5 cm from top). Two samples were placed on one plate and 2 plates were used for 3 parallels and for control.

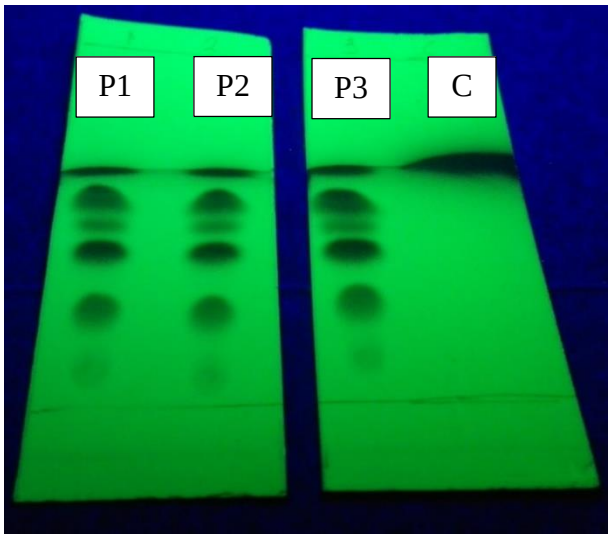


Figure 18. Image of the TLC Plates from Reaction Mixture.

Preparation of the reaction mixture for the seven day analysis

The prepared mCPBA (8 mL) solution was slowly added to the DMTS (32 mL) solution in the reaction flask between -5° and 0° C. It was stirred for 30 minutes using a glass rod. Then the reaction mixture was kept at room temperature for an hour. 1200 µL of reaction mixture was aliquoted to each HPLC vial and kept at room temperature for further analysis. 21 vials were prepared to yield 3 parallels for each consecutive day.

Preparation of Day 0 to Day 7 samples for HPLC and GC-MS analysis

Sample of the reaction mixture (500 µL) was vortexed and extracted with 500 µL carbonate buffer solution in an eppendorf vial. The organic layer was saved. The sample from organic layer (50 µL) was diluted with 450 µL ethanol and was autovortexed for 5 minutes. Sample solution (70 µL) was mixed with 630 µL of ethanol and hand vortexed for 30 seconds. This sample was used for the HPLC, TLC and the GC-MS analysis. No internal standard was added during the preparation of the samples for HPLC UV-Vis, TLC and GC-MS analysis.

Analysis of Day 0 to Day 7 samples on TLC plates

Sample (5 µL) was placed on the prepared TLC plate 0.5 cm above the running solvent level. Two drops were placed on one plate and the solvent was let to evaporate. A thin layer of mobile phase (hexane) was poured into the base of a small beaker. The spotted TLC plates were placed into the beaker. The whole setup was covered with a larger beaker to let the system to saturate with solvent. The TLC plate was removed from the apparatus just before the solvent front reached the end of the plate.

The separation of components on the TLC plates were observed under law wavelength (254 nm) UV-Light. The observed component spots were marked as polar and non-polar for further analysis.

Preparation of Reactants for DMTS Oxidation with H_2O_2

Preparation of DMTS solution in 15% poly 80

Poly 80 (1.5 g) was added to a 10 ml volumetric flask, filled up with distilled water and vortexed for 5 min to prepare 15% (m/v) poly 80 solution. DMTS (50 mg/mL) was prepared in 15% (m/v) poly 80 solution.

Preparation of 3% H_2O_2 solution

H_2O_2 (800 µL) was added to 7200 µL of distilled water and vortexed.

Preparation of the reaction mixture for DMTS Oxidation with H_2O_2

H_2O_2-3% (v/v) (500 µL) was added to 500 µL 50 mg/ml DMTS in 15% (m/v) poly 80. The reaction mixture was separated into 15 vials and kept for 5-days at room temperature.

Preparation of the Samples for GC-MS Analysis, Separated from TLC Plates

The identified TLC sample spots were cut into tiny pieces and placed in an eppendorf vial. ACN or acetone (150 µL) was added and the vial was auto-vortexed for 2 minutes. Then the eppendorf vial was centrifuged at 14000 *g* for 5 minutes. 50 µL of the resulting solution was transferred into vial and 1 µL was injected to the GC-MS.

Preparation of F3-Formulated DMTS and Pure DMTS for Oxidation Comparison Study.

The DMTS (0.063 g) was dissolved in 5 mL of CH_2Cl_2 to prepare 0.1 M pure DMTS solution. F3-formulated DMTS (315 μL) was dissolved in 685 μL of CH_2Cl_2 and 100 μL of it was diluted with 900 μL of CH_2Cl_2 to prepare 0.1 M F3-formulated DMTS. The mCPBA (0.086 g) was dissolved in 5 mL of CH_2Cl_2 to prepare 0.1 M mCPBA solution. The prepared solutions were kept between -5 and 0 ºC until the reactions began.

400 μL of 0.1 M pure DMTS and 400 μL 0.1 mCPBA were reacted in a HPLC vial and similarly 400 μL of 0.1 M F3-formulated DMTS and 400 μL 0.1 mCPBA were reacted in a separate HPLC vial. These were let to react for one week and analyzed in HPLC using previously mentioned instrumental method for F3-formulation analysis.

CHAPTER III

RESULTS AND DISCUSSION

PART 1

Storage of the Formulated DMTS

Previously, three methods of storage were studied for their stability. Those studied were: a) single crimp-seal method, b) double crimp-seal method and c) hermetically fire-sealed glass ampule method. Storage sealing method was compaired for F2-formulated DMTS, and the hermetically fire-sealed glass ampules proved to be the best storage method. With the single crimp-seal method, the F2-formulated DMTS was stable for only two days and after one-week only 40% (M/M) of the original DMTS was detected. With the double crimp-seal method after two weeks, 65% (M/M) of the original DMTS amount was detected. However, the hermetically fire-sealed glass ampule stored samples showed good stability: the Poly80 DMTS solution was stable for a month with 100% (M/M) recovery.[28] Therefore, the ampule method was chosen to study the long-term storage stability of the F3-formulated DMTS.

Determination of Sample Condition Before and After Ampule Sealing

Here, 1900-2000 ºC flame was used to seal the ampules, which can cause damage by thermally reacting with the samples inside the ampules. It was important to check if there was any change in the DMTS content during the ampule sealing. Therefore, three samples were analyzed before and after ampule sealing using the previously set instrumental method. Results showed that the flame had no effect on the formulated DMTS during fire sealing (Figure 19).

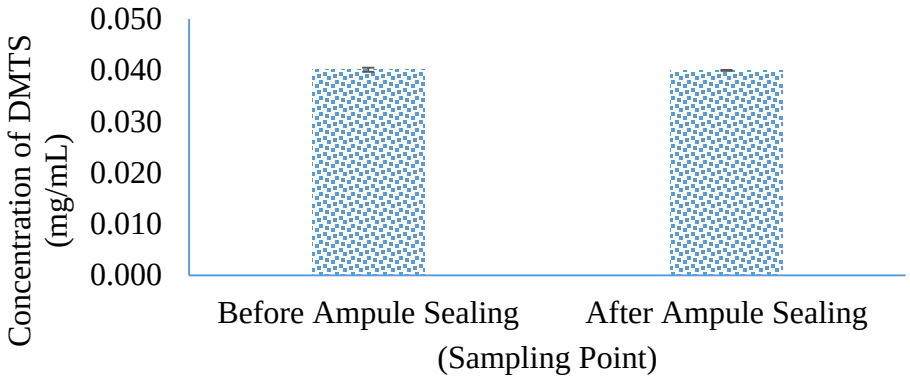


Figure 19. Concentration Comparison of F3-formulated DMTS Before and After Sealing of Glass Ampules. Data are presented as mean ± S.D., n = 3.

The specific wavelength used in the HPLC-UV/Vis for DMTS determination was 215 nm. The intensity of DMTS and DMDS have gone up at lower wavelengths. The selected wavelength was used because the solvent cut-off wavelength in HPLC for acetonitrile is 200 nm. The following are the UV-Vis spectra for DMTS and DMDS (Figure 20). The storage temperatures, which were 4 ºC in refrigerator, 22 ºC in cupboard and 37 ºC in oven, did not show any significant fluctuation at the sampling point throughout study period.

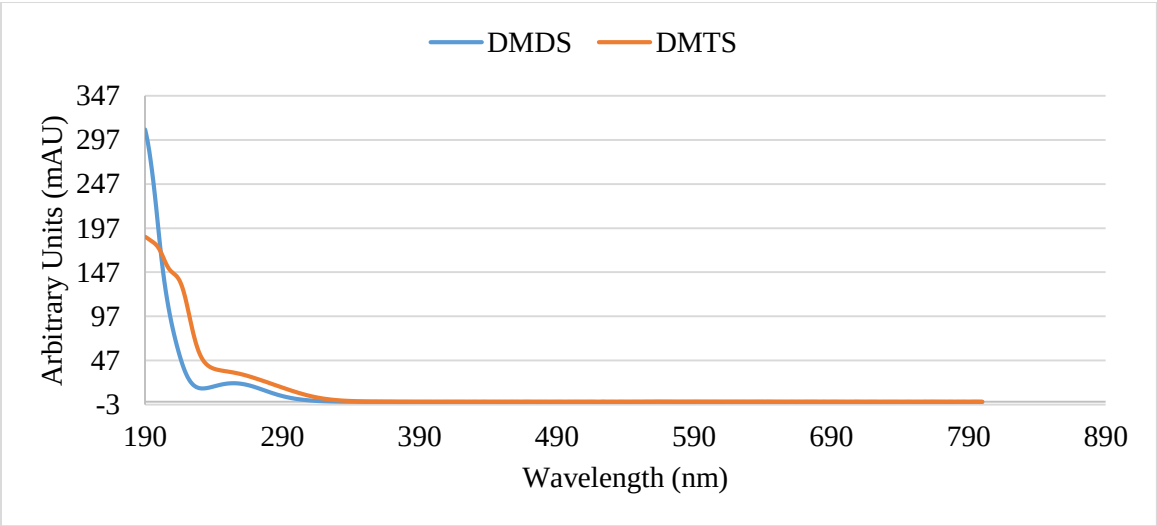


Figure 20. UV-Vis Spectra Corresponds to the DMTS Analysis in HPLC.

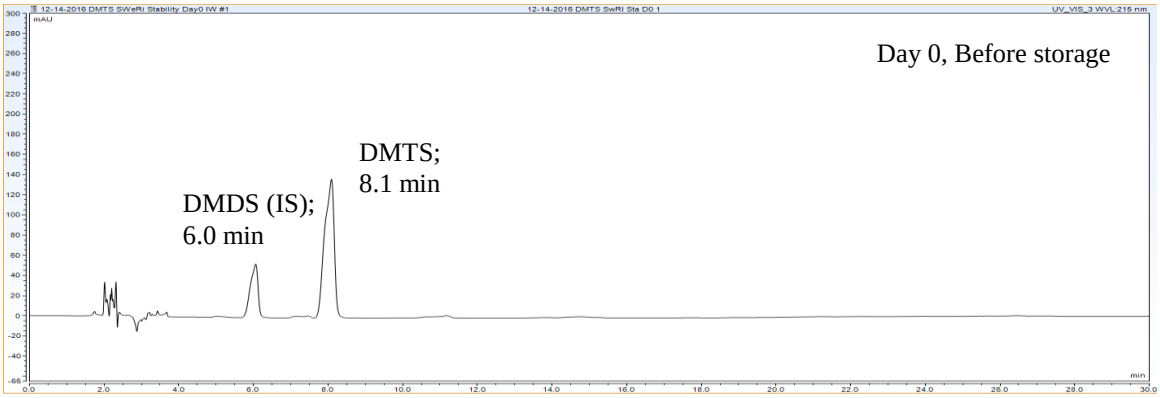


Figure 21. HPLC Chromatogram of the F3-Formulated DMTS (1:15000 diluted) on Day 0. The internal standard was DMDS (0.1 mg/mL in ACN).

In the spectrum, the retention time for DMTS was 8.1 min and for the internal standard (IS) DMDS 6.0 min (Figure 21). The peak areas of DMTS and DMDS were used to calculate the peak area ratios, with following formula (Equation 1).

$$Peak\ Area\ Ratio = \frac{Peak\ Area\ of\ DMTS\ (mAU{*}min)}{Peak\ Area\ of DMDS\ (mAU{*}min)}$$

Equation 1: Formula for the Calculation of Peak Area Ratio.

All the sampling points were analyzed with the aid of a calibration curve which gave good linearity with increasing concentration (Figure 22). The calibration curve showed the equation of $y = 102.23x - 0.0433$ with the $R^2 = 0.9991$.

The limit of detection (LOD) and limit of quantification (LOQ) were determined for the method.

LOD $= Y_{blank} + 3s = 0.0238 + (3 \times 6.98{*}10^{-5}) = 0.0240$

Concentration LOD = 3s/ m $= (3 \times 6.98{*}10^{-5})/102.23 =$ ***2.05 ng/mL***

LOQ $= Y_{blank} + 10s = 0.0238 + (10 \times 6.98{*}10^{-5}) = 0.0245$

Concentration LOQ = 10s/m $= (10 \times 6.98{*}10^{-5})/102.23 =$ ***6.83 ng/mL***

Where Y_{blank} is the mean of the blank signal, *s* is the standard deviation of the signal for the lowest analyzed concentration and *m* is the gradient of the calibration curve.

$s = \sqrt{\frac{\sum(x_m - x_i)^2}{n-1}}$ where x_m is the mean of the repeats, x_i is the value of an individual measurement and *n* is the number of repeats for the lowest analyzed concentration.

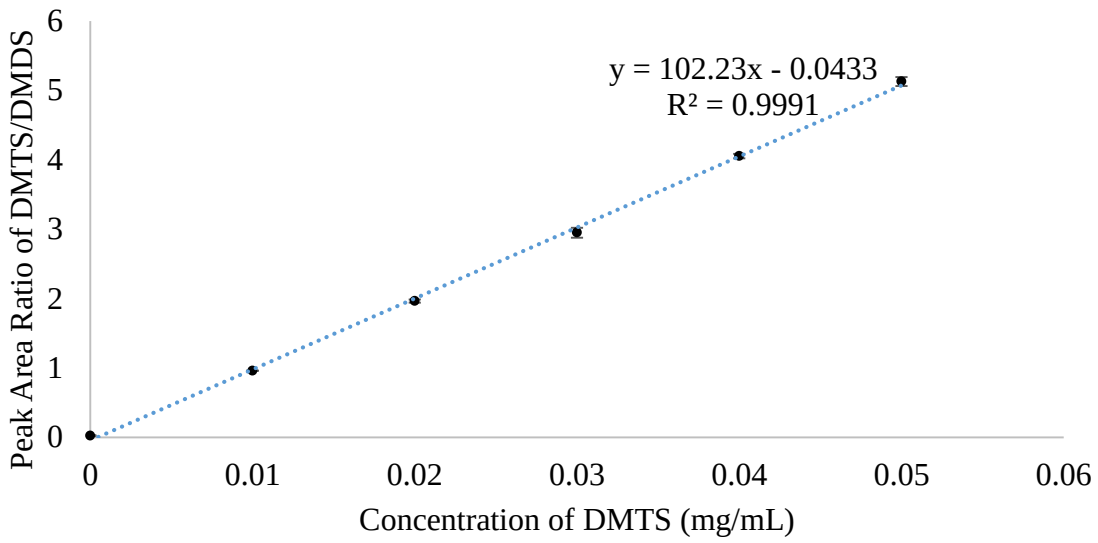


Figure 22. Calibration Curve Prepared for the Determination of the DMTS Concentration of the Stability Samples (400 mg/mL DMTS in the F3-Formulation). *Data are presented as mean ± S.D., n=5. Some error bars are not visible as the S.D. is low.*

The intra- and inter-day precisions and accuracies were estimated using concentrations of DMTS in F3-formulation (Table 2). The intra- and inter-day precisions varied from 0.19 to 2.34 CV%, while the intra- and inter-day accuracies varied from -2.37 to 1.18 %. According to the FDA guidelines, both precision and accuracy should fall within a range of ±15%.[49] Following equations were used to calculated precision and accuracy (Equaiton 2, Equation 3).

$$Precision = \frac{Std.\,Deviation\ of\ the\ Measured\ Concentration}{Mean\ of\ the\ Measured\ Concentration} \times 100\%$$

Equation 2: Formula for Calculation of Precision.

$$Accuracy = \left(\frac{Std.\,Deviation\ of\ the\ Measured\ Concentration}{Mean\ of\ the\ Measured\ Concentration} \times 100\%\right) - 100$$

Equation 3: Formula for Calculation of Accuracy.

Table 2.

Precision and Accuracy Values for the F3-Formulated DMTS Quantification.

Nominal Concentration (µg/mL)	Measured Concentration (µg/mL)	Standard Deviation of the Concentration ±*10^{-4}	Precision (CV%)	Accuracy (Bias, %)
Intra-day (n=5)				
10.0	9.815	0.19	0.20	-1.85
20.0	19.62	2.13	1.09	-1.89
30.0	29.26	6.90	2.36	-2.46
40.0	40.05	3.14	0.78	0.13
50.0	50.58	6.24	1.23	1.17
Inter-day (n_{day1} = 5, n_{day2} = 5)				
10.0	10.12	0.93	0.92	1.19
20.0	20.08	3.09	1.54	0.40
30.0	30.10	4.29	1.42	0.35
40.0	40.00	6.37	1.59	0.01
50.0	49.88	1.50	0.30	-0.24

Determination of the Long-term Storage Stability of the F3-Formulated DMTS

The objective of this study was to determine the long-term storage stability of the F3-formulated DMTS contained in hermetically fire-sealed glass ampules. Since the previous studies showed significant protection for the F2-formulated DMTS in hermetically fire-sealed glass ampules, the same sealing approach was used for the current study. Samples were allowed to come to room temperature prior to analysis (since the

samples stored at different temperatures). The necks of the glass ampules were broken with gloved hand with proper care. All the sampling points were analyzed after 15000 times dilution in HPLC-UV/Vis and the concentration of each sample was calculated using the calibration curve (*Figure 22*). The % (M/M) DMTS was calculated using the following equation (Equation 2).

$$\%\ DMTS = \frac{Concentration\ of\ the\ DMTS\ at\ Sampling\ Point}{Concentration\ of\ DMTS\ before\ Storage\ (Time\ 0)} \times 100\%$$

Equation 4: Formula for the Calculation of Percent DMTS.

The results showed that there was no measurable loss of DMTS over the one-year period of the study for the samples stored at 4, 22 ºC. The samples stored at 37 ºC showed good stability for five months (Figure 23). The data used to calculate the % DMTS during the study are included in the appendix B. Some of the replicate data from the data points were removed due to dilution errors.

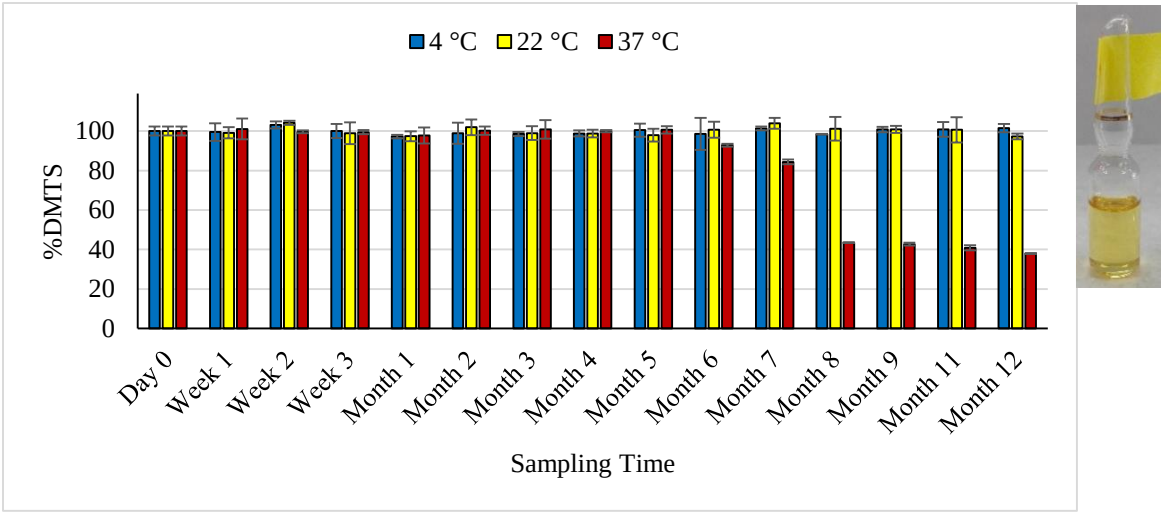


Figure 23. One Year Stability of the DMTS in the F3-Formulation. *Data are presented as mean ± S.D., n=2-3. Some error bars are not visible as the S.D. are low.*

Since the concentration change in the sixth-month sample at 37 ºC was significant, the HPLC spectra (Figure 25) were examined for any differences in the peak heights and peak areas. The solutions were observed for any color and physical changes. It was also significant that the 37 °C stored sample changed its color: the light yellow color has changed to dark brown color. The solution was transparent; no precipitation was observed (Figure 24).

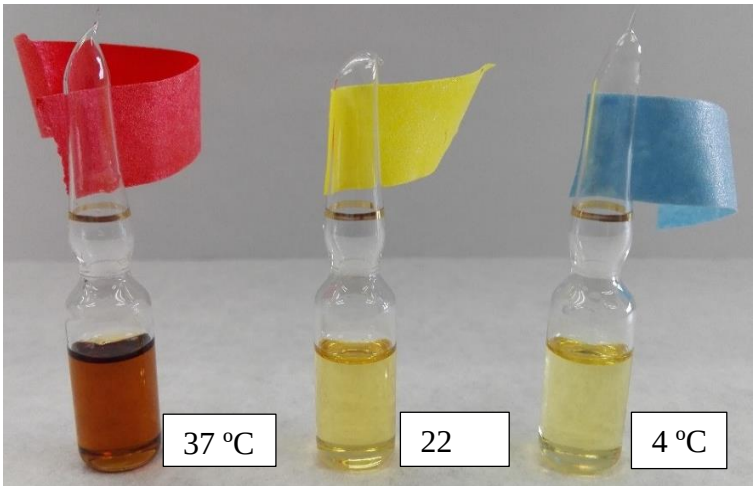
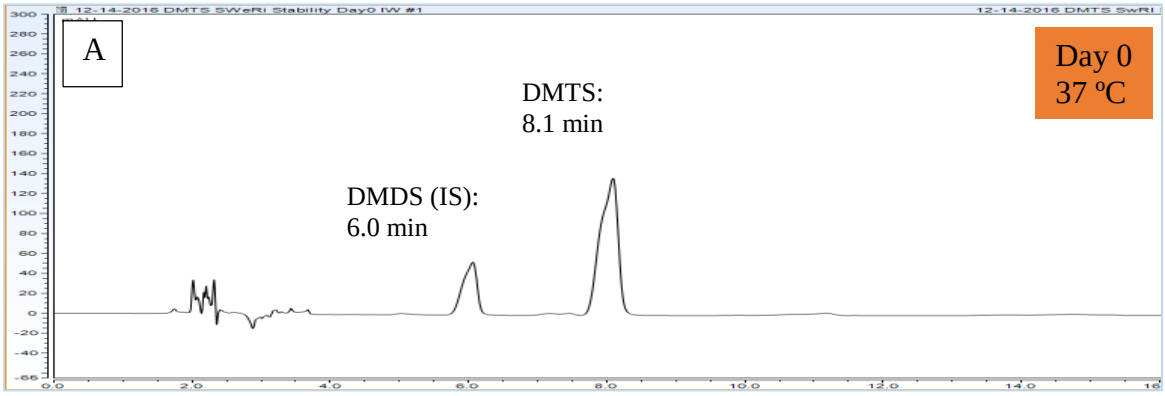
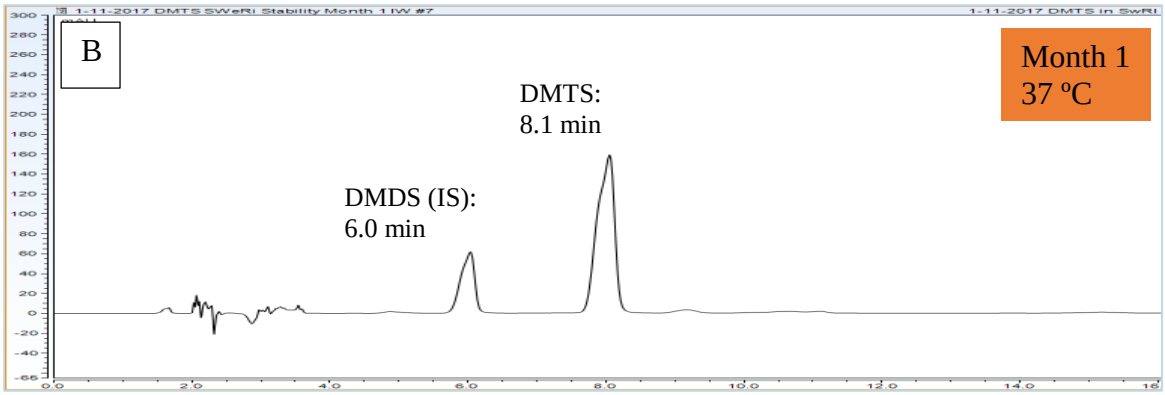
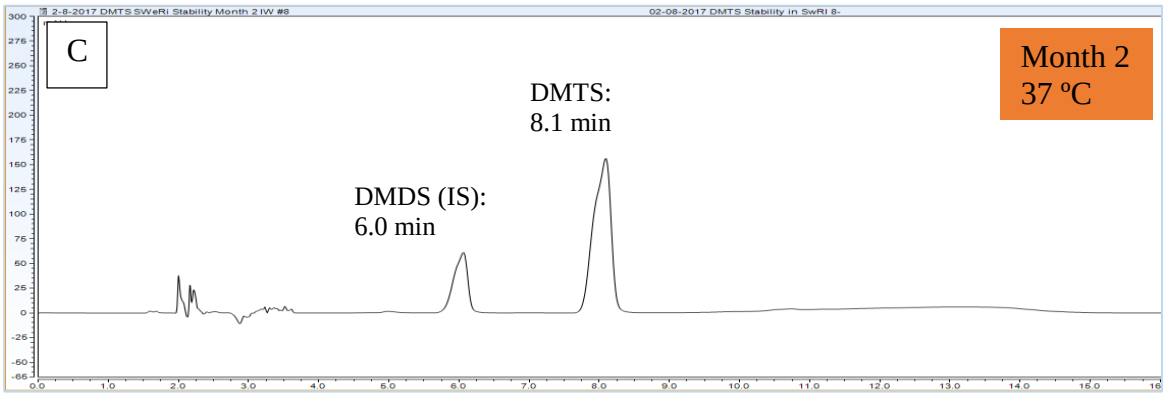


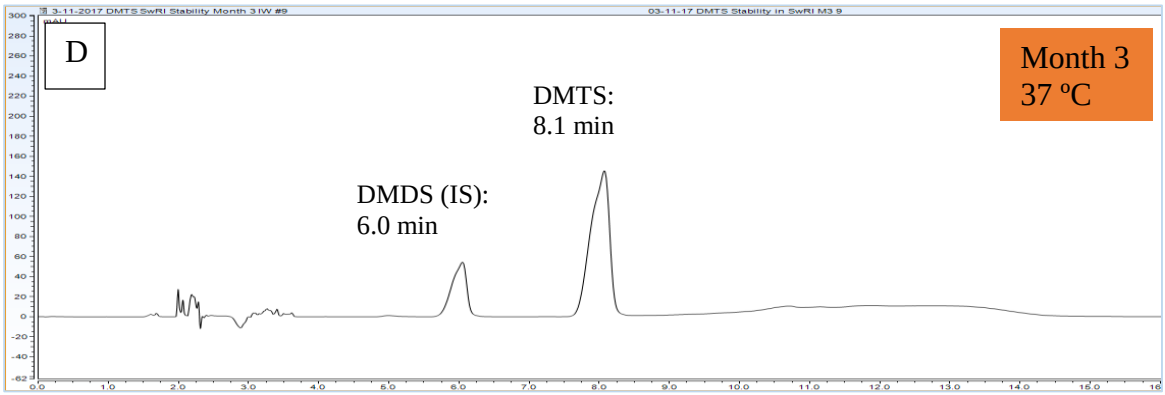
Figure 24. Color Change of the F3-Formulated DMTS stored at 37 ºC in an Ampule at Month 7 Compared to the Samples Stored at 4 and 22 ºC.

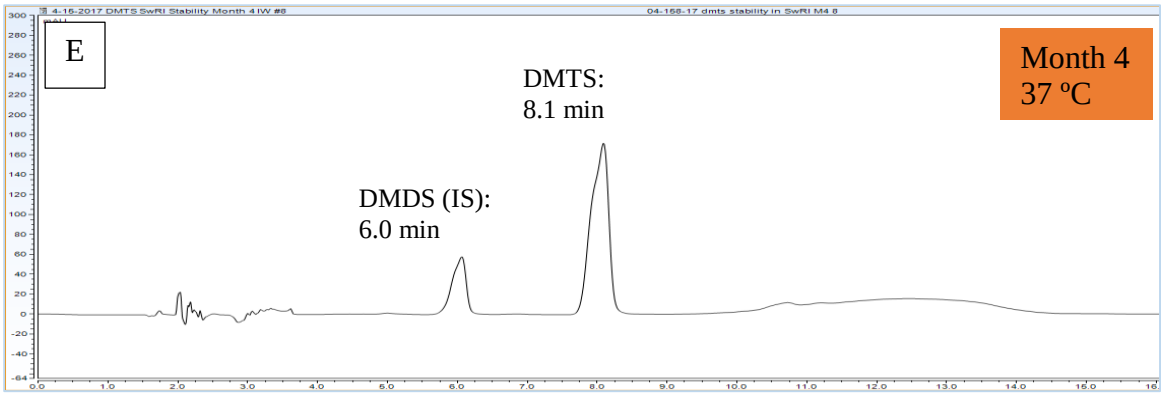
All the spectra were compared from Month 0 to Month 12 at 37 ºC to identify the growth of the new peaks and the decrease of the DMTS peak in all chromatograms (Figure 25).

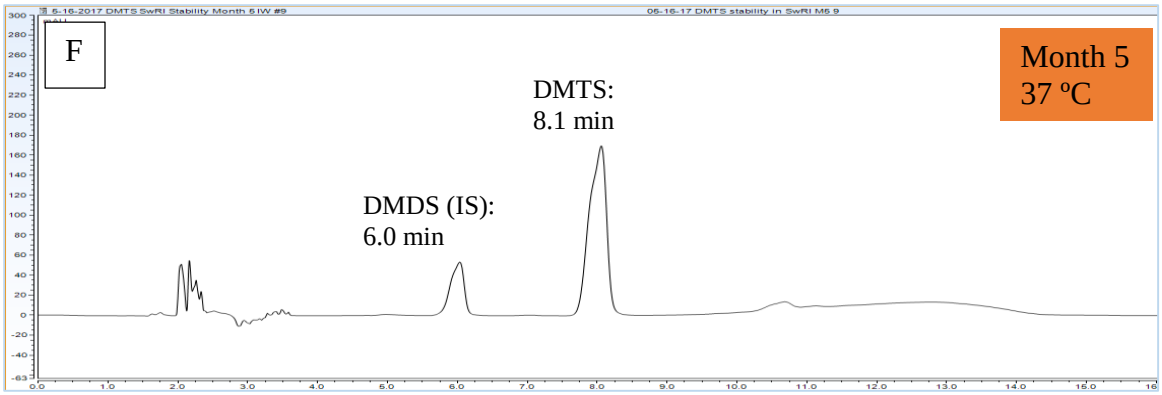


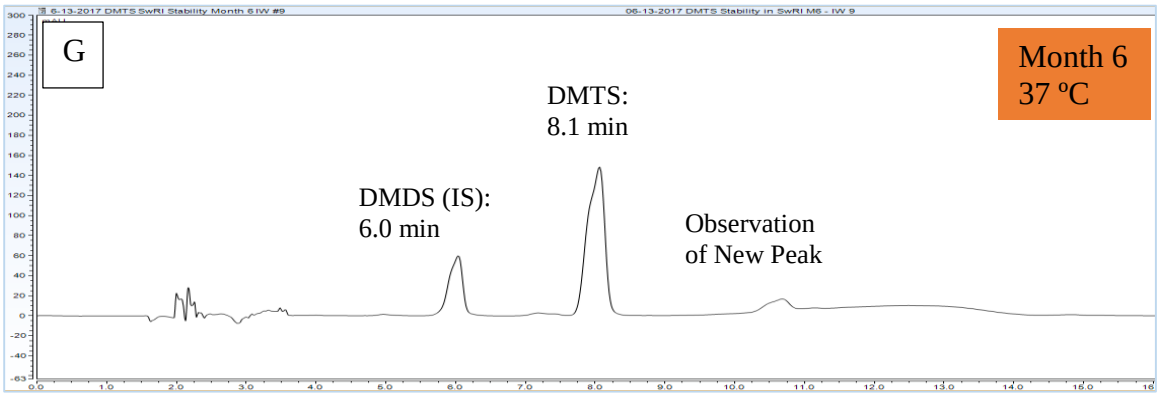

B
Month 1
37 ºC
DMTS:
8.1 min
DMDS (IS):
6.0 min

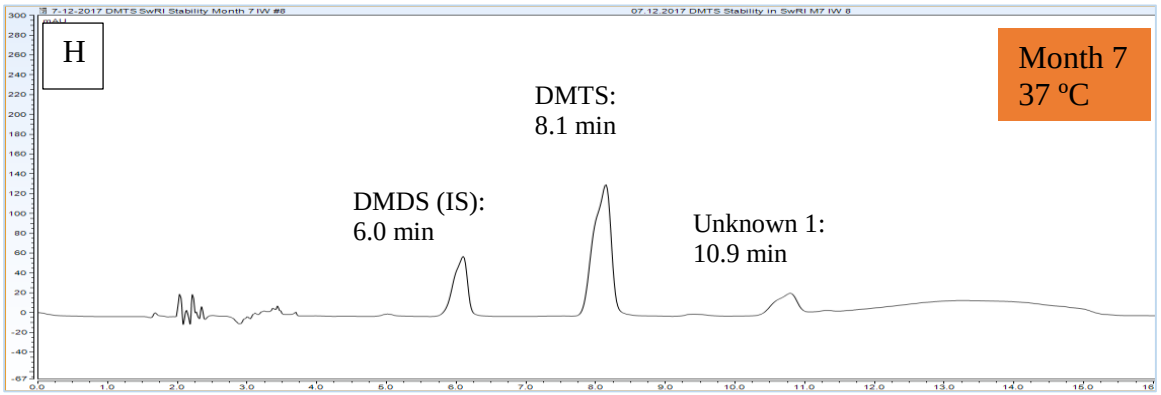

C
Month 2
37 ºC
DMTS:
8.1 min
DMDS (IS):
6.0 min

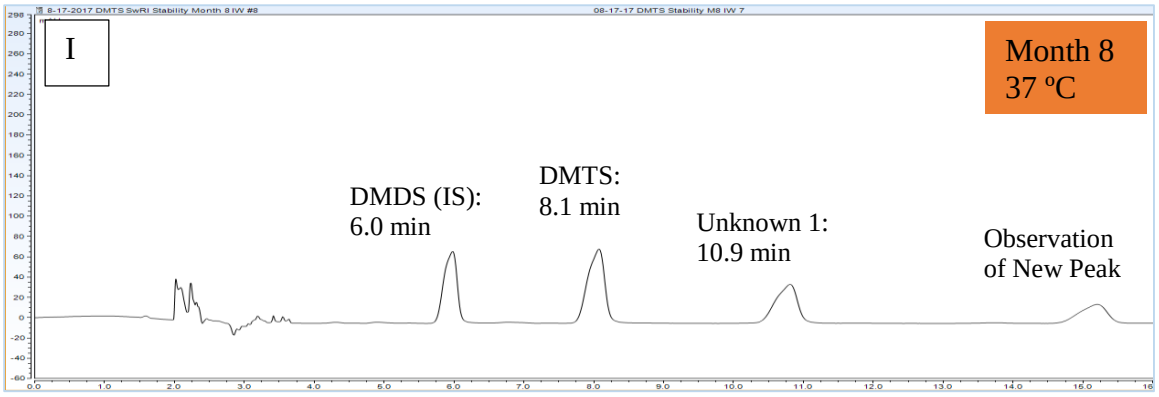

D
Month 3
37 ºC
DMTS:
8.1 min
DMDS (IS):
6.0 min


E
Month 4
37 ºC
DMTS:
8.1 min
DMDS (IS):
6.0 min


F
Month 5
37 ºC
DMTS:
8.1 min
DMDS (IS):
6.0 min


G
Month 6
37 ºC
DMTS:
8.1 min
DMDS (IS):
6.0 min
Observation
of New Peak


H
Month 7
37 ºC
DMTS:
8.1 min
DMDS (IS):
6.0 min
Unknown 1:
10.9 min


I
Month 8
37 ºC
DMDS (IS):
6.0 min
DMTS:
8.1 min
Unknown 1:
10.9 min
Observation
of New Peak

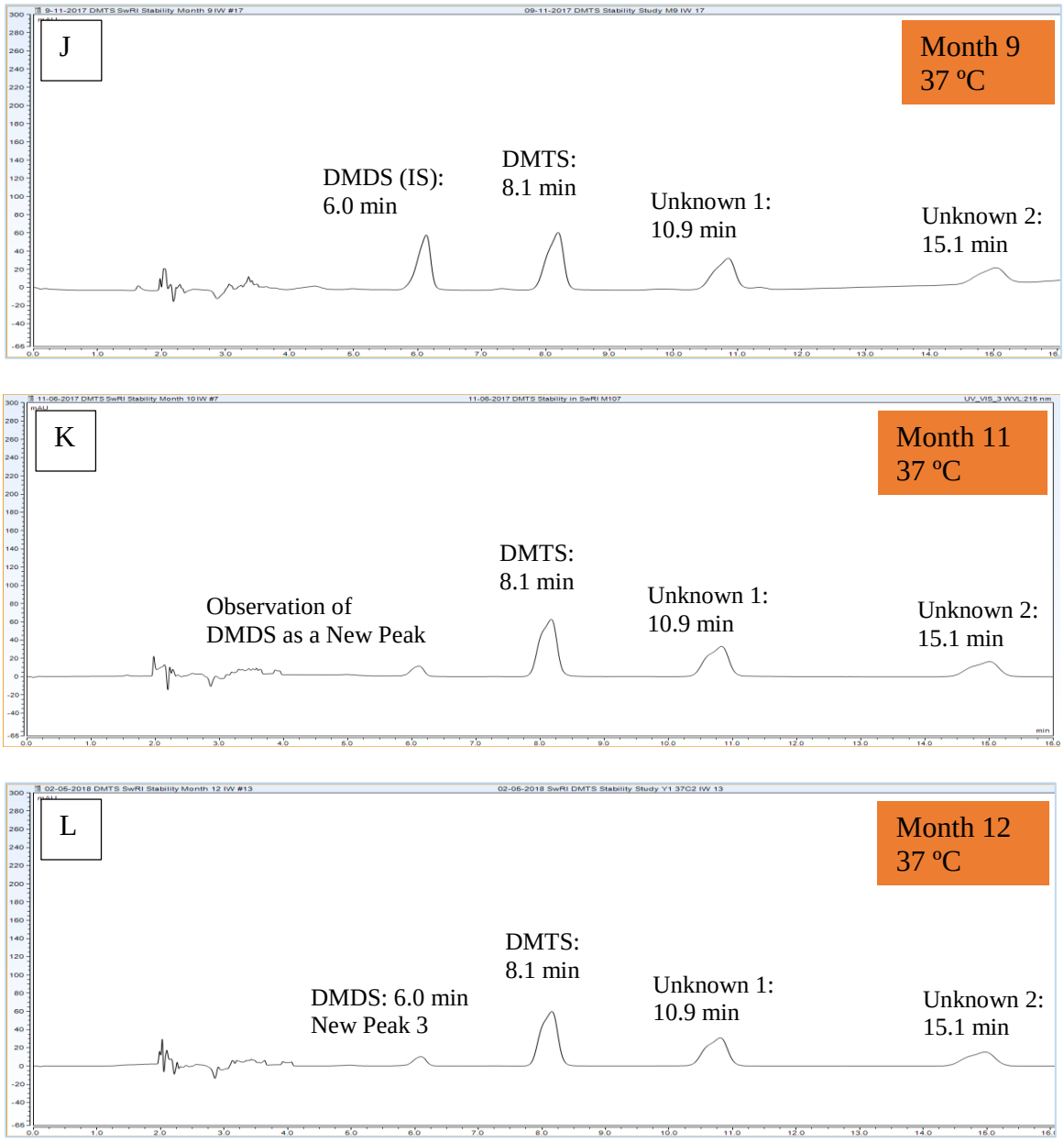


Figure 25. Chromatograms of the F3-Formulated DMTS Stored at 37 ºC. A-L are from time 0 to year 1. *Mobile phase used 65:35 = ACN:H_2O*

Observations of the chromatogram from month 6 showed the development of a new peak at 10.9 min retention time and the chromatogram from month 8 showed development of another peak at 15.1 min. These peaks were labelled as unknown 1 and unknown 2 until further analysis. Due to the curiosity of the observations month 9 sample was analyzed in

HPLC-UV/Vis without IS DMDS and the chromatogram showed a peak at 6.0 min suggesting that degradation of DMTS helped to develop DMDS as one of the degradation product (Figure 26). Product DMDS peak labeled as New Peak 3. The height and area of DMTS peak have gone down significantly while the peak area and the height have gone up for DMDS (product), Unknown 1 and Unknown 2. With the occurrence of new the peaks the formulation got darker. For further clarification, chromatograms obtained from samples at 37 ºC, those were plotted in a overlay graph (Figure 27). The board peak observed in the chromatograms could not be identified as one of the product since in has shown in all the three different temperatures except for the very first injection. It was considered as contamination of the column form the previous injection.

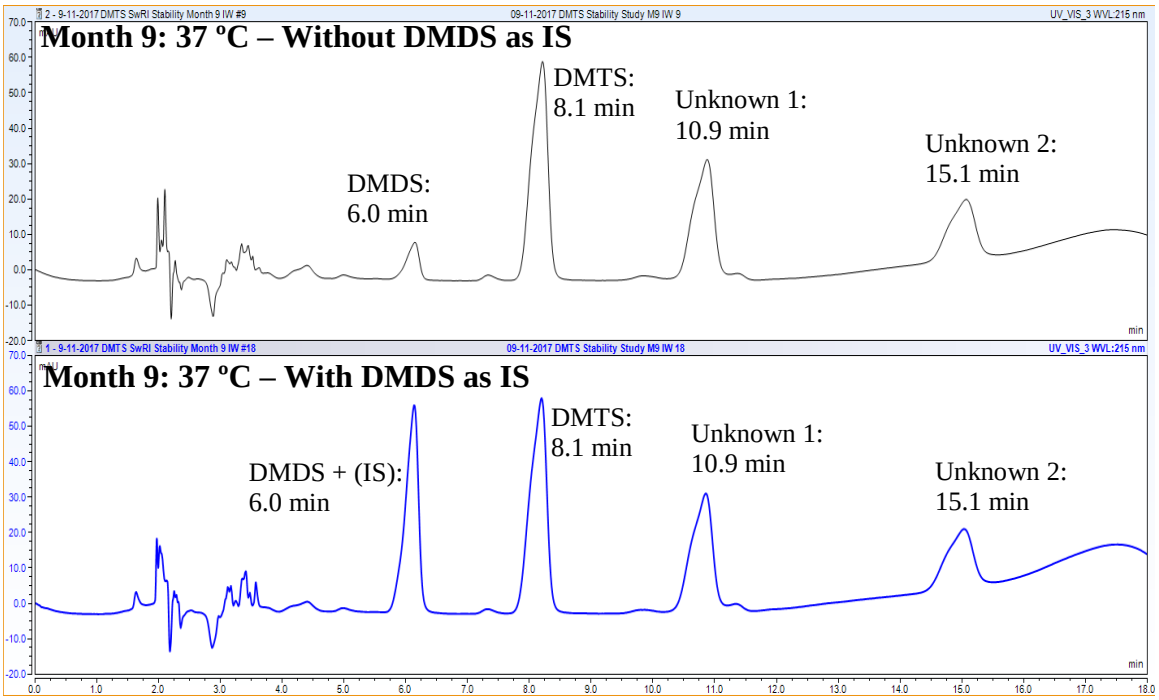


Figure 26. Comparison of Chromatograms of the 37 ºC Stored Month 9 F3-Formulated DMTS sample with and without internal standard. *Mobile phase used 65:35 = ACN:H_2O*

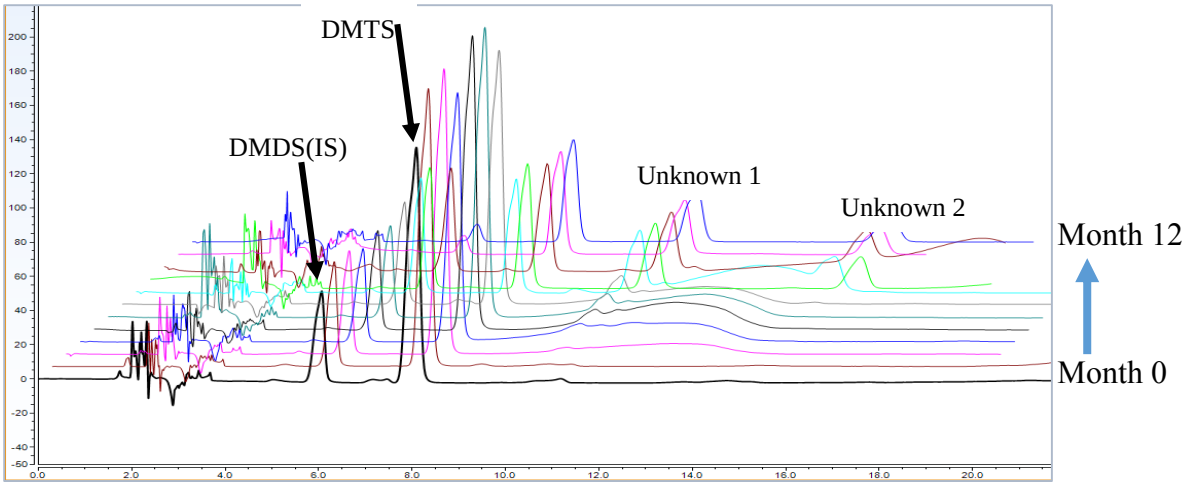
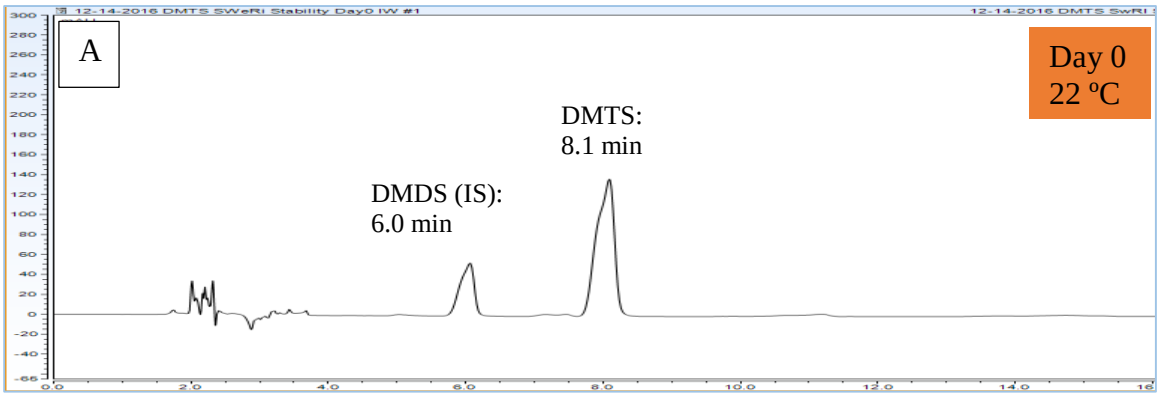
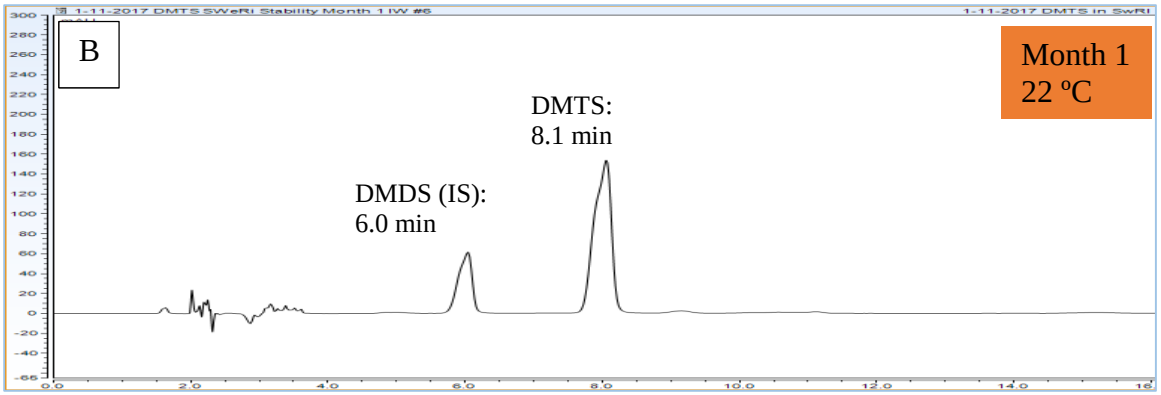
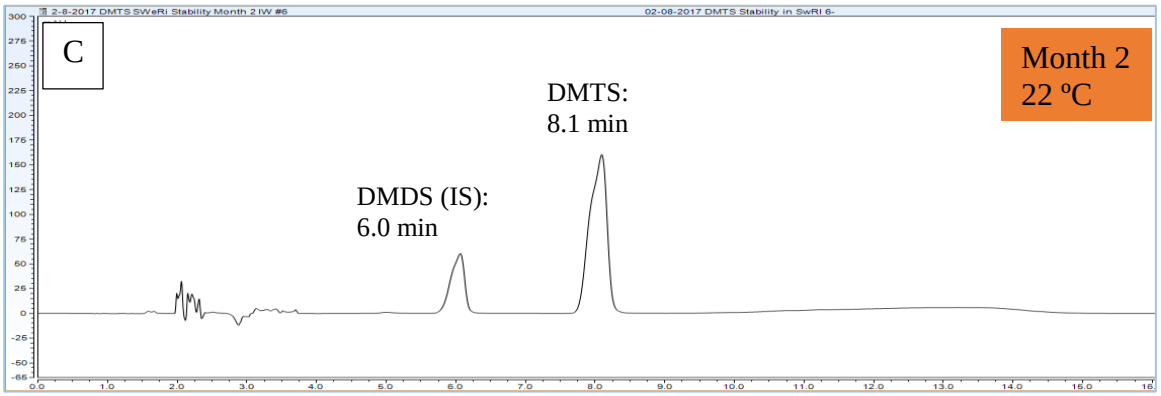


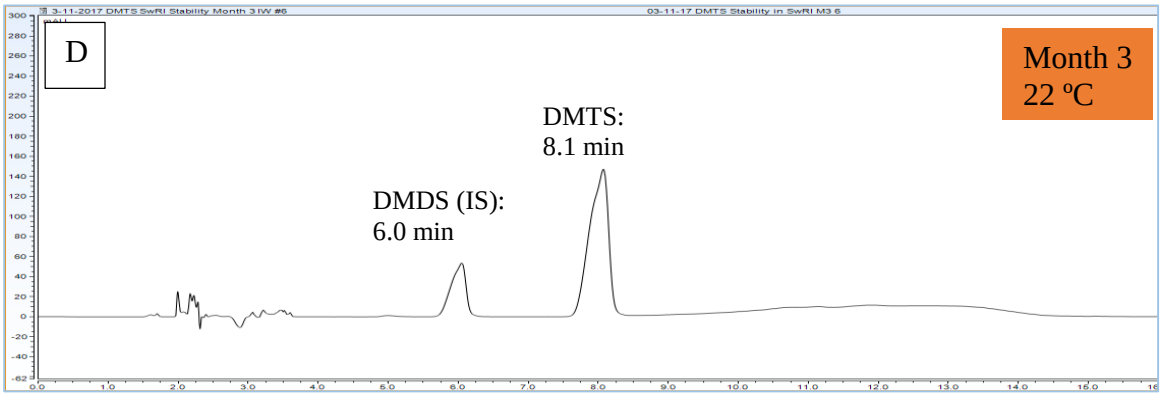
Figure 27. HPLC Chromatograms of the Samples of F3-Formulated DMTS up to Month 12 Stored at 37 ºC. *Mobile phase used 65:35 = ACN:H_2O*

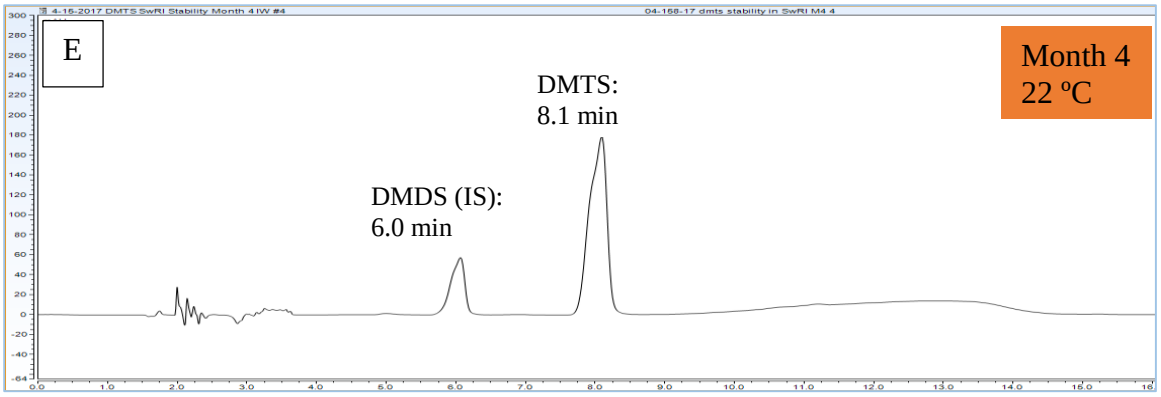
This observation lead to the examination of the color changes in other samples stored at 22 ºC temperature. There was no color change in the samples stored at 22 ºC. The color change of the samples were examined by nacked eye. The chromatograms were inspected for occurance of new peaks at same retention times. The chromatograms obtained are shown in Figure 28.

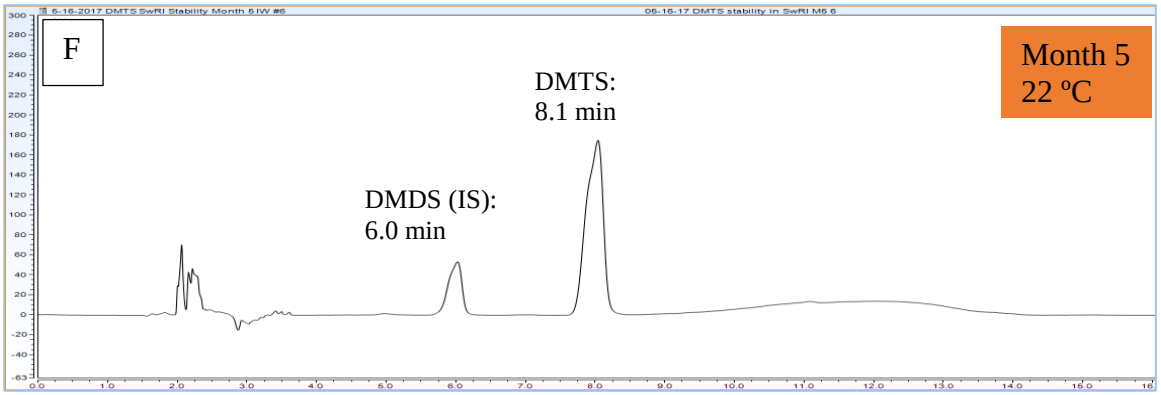


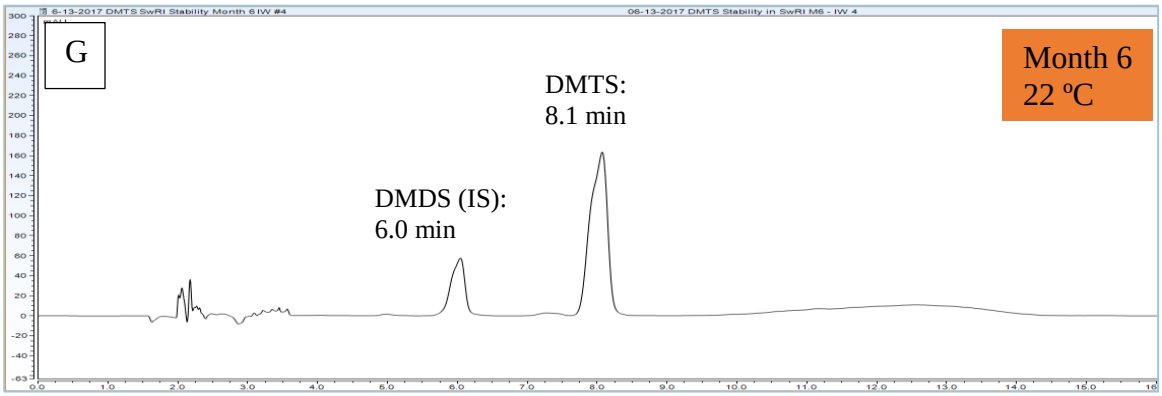

B
Month 1
22 ºC
DMTS:
8.1 min
DMDS (IS):
6.0 min

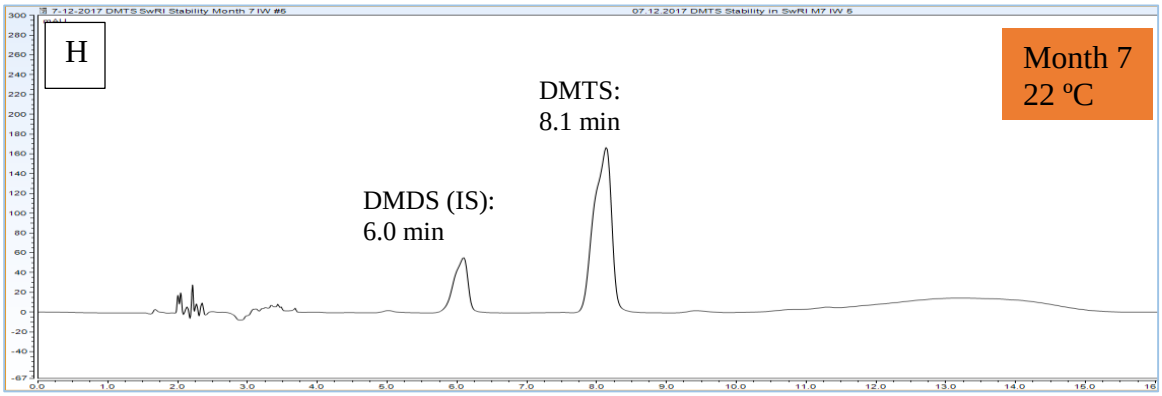

C
Month 2
22 ºC
DMTS:
8.1 min
DMDS (IS):
6.0 min

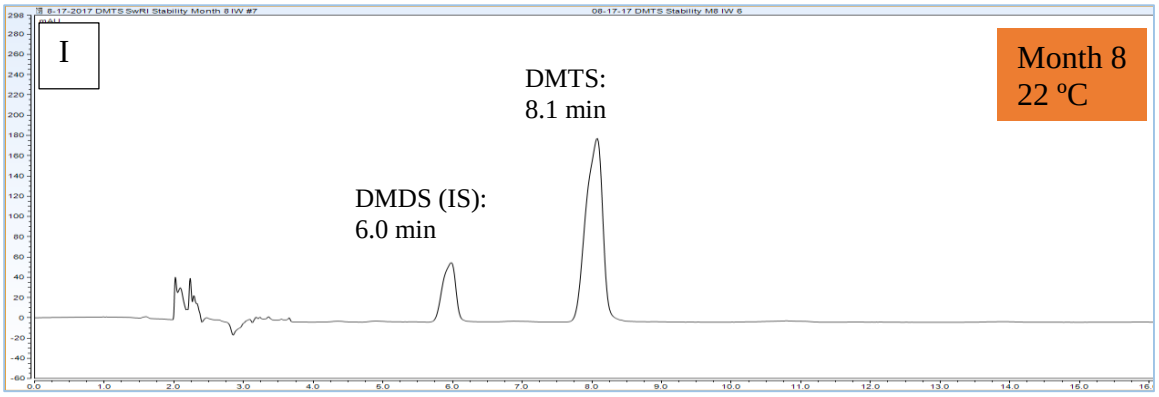

D
Month 3
22 ºC
DMTS:
8.1 min
DMDS (IS):
6.0 min


E
Month 4
22 ºC
DMTS:
8.1 min
DMDS (IS):
6.0 min


F
Month 5
22 ºC
DMTS:
8.1 min
DMDS (IS):
6.0 min


G
Month 6
22 ºC
DMTS:
8.1 min
DMDS (IS):
6.0 min


H
Month 7
22 ºC
DMTS:
8.1 min
DMDS (IS):
6.0 min


I
Month 8
22 ºC
DMTS:
8.1 min
DMDS (IS):
6.0 min

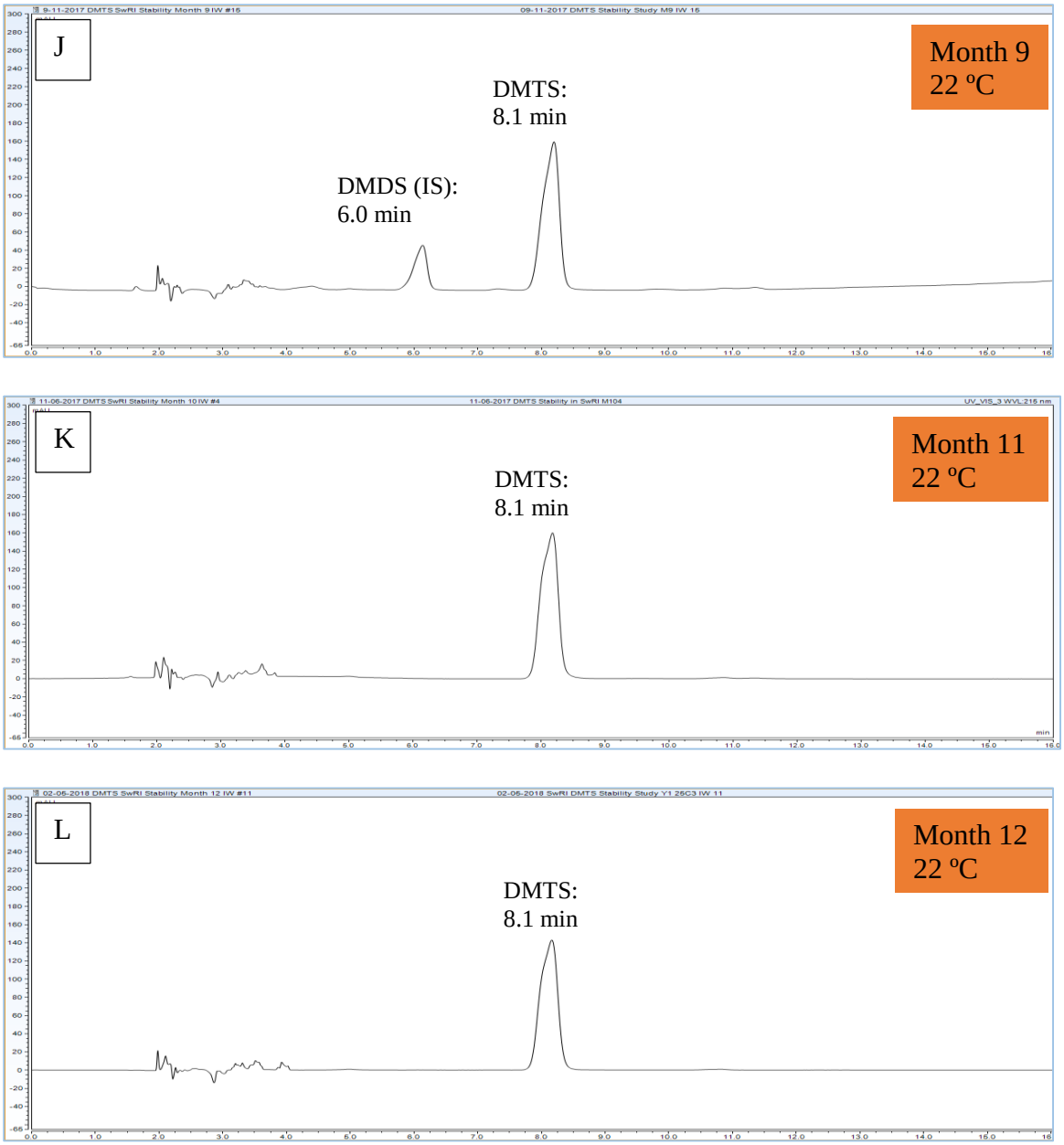


Figure 28. Chromatogram Comparison of the F3-Formulated DMTS Stored at 22 ºC from Time 0 to Year 1. *Mobile phase used 65:35 = ACN:H_2O*

The chromatograms obtained from samples stored at 22 ºC showed no observable new peaks throughout the period of analysis suggeting that the samples are stable at room temperature. The chromatograms from the samples stored at 4 ºC and 22 ºC were also observed for spectral differences occuring as a funtion of time (Figure 29).

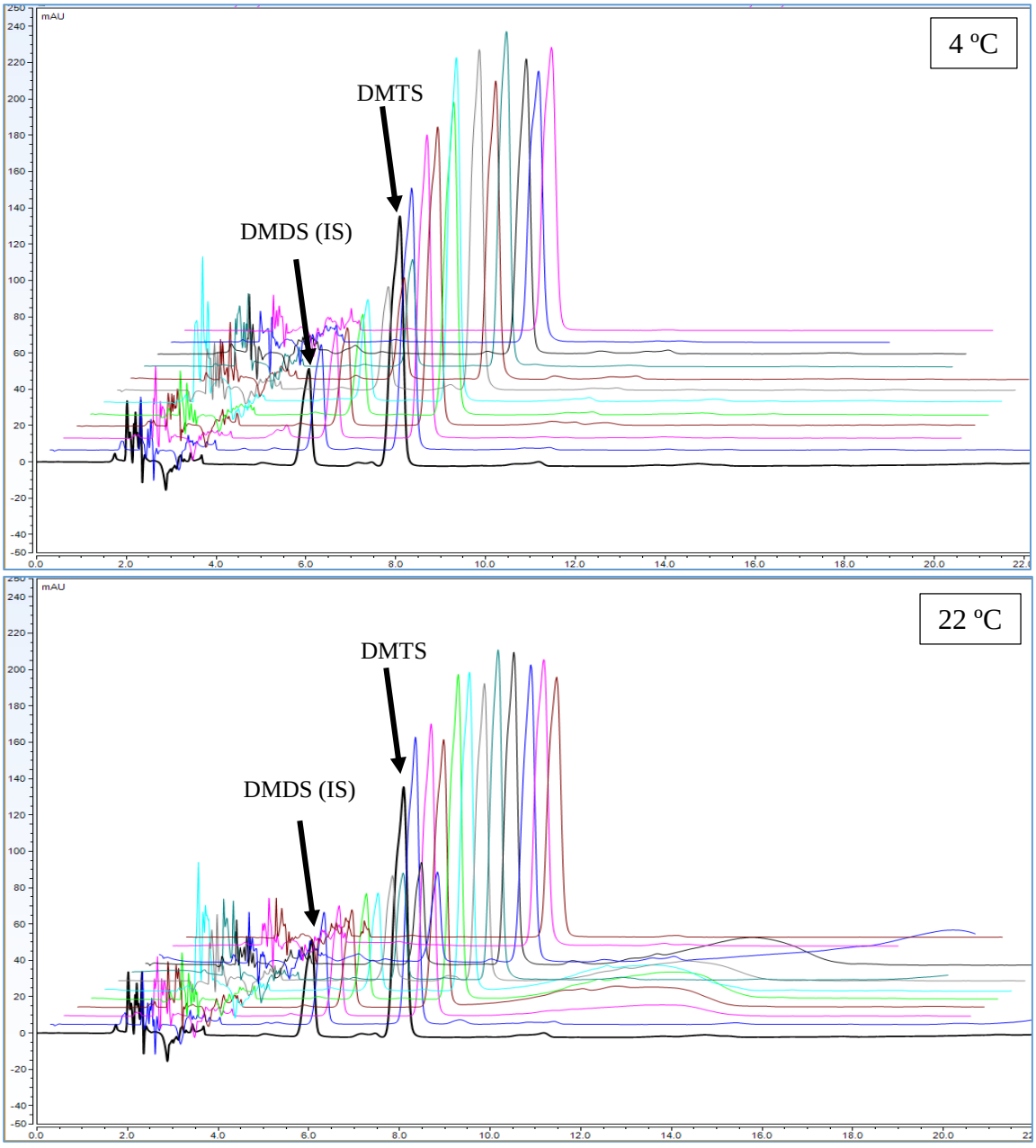


Figure 29. Chromatograms of the F3-Formulated DMTS up to Month 12 Samples Stored at 4 ºC and 22. *Mobile phase used 65:35 = ACN:H_2O.*

According to the chromatograms obtained from samples stored at 4 and 22 ºC, there were no new peaks. These observations confirm the long-term stability of F3-formulated DMTS in samples stored at 4 and 22 ºC conditions. The solvent run time was changed at month 2 to 20 min and at month 8 to 18 min to save the wastage of solvents.

Identification of the Degradation Products.

Observation of new peaks led to the investigation on degradation products. A GC-MS SPME method was used in this study which can provide good filtering mechanism which ensures, only volatile components are injected to the GC-MS, because the polymer surfactants are very nonvolatile and would risk plugging the analytical column. It needed to use high split ratio due to the high concentration of these samples even after degradation. After several set of preliminary experiments, the optimum split ratio of 600:1 was selected. According to the MS library, three major components were identified with 90% top hit match for each of the compound. There was only one significant peak for the samples at low temperatures (Figure 30). The identified products are DMDS, DMTS and DM4S with the retention times of 2.20 min, 3.05 min and 3.90 min, respectively (Figure 31). There was no degradation at the injection port according to the GC parameters used. This confirms the occurrence of identified products at higher temperature stored samples.

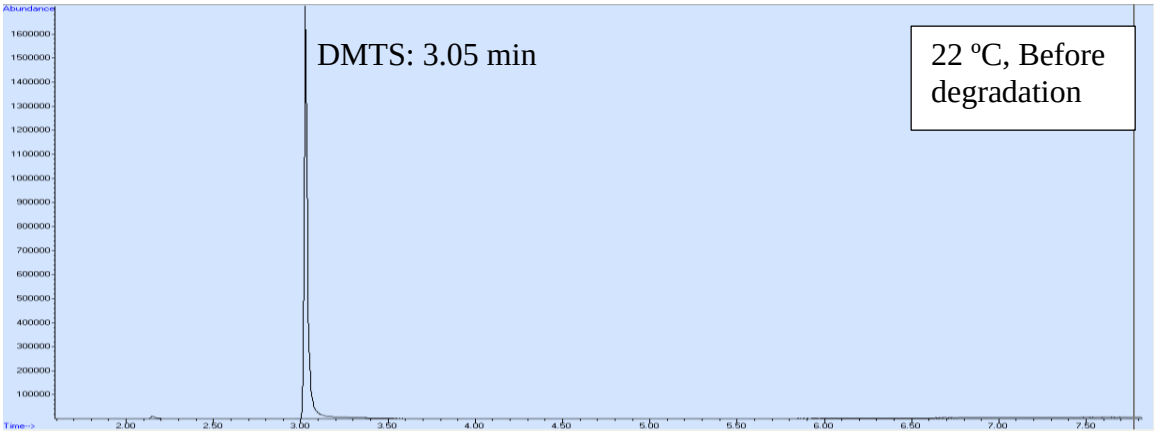


Figure 30. GC Chromatograms of the F3-Formulated DMTS Stored at 22 ºC at Month 11. *600:1 split ratio was used in the SPME method (30 s) and He was used as the carrier gas.*

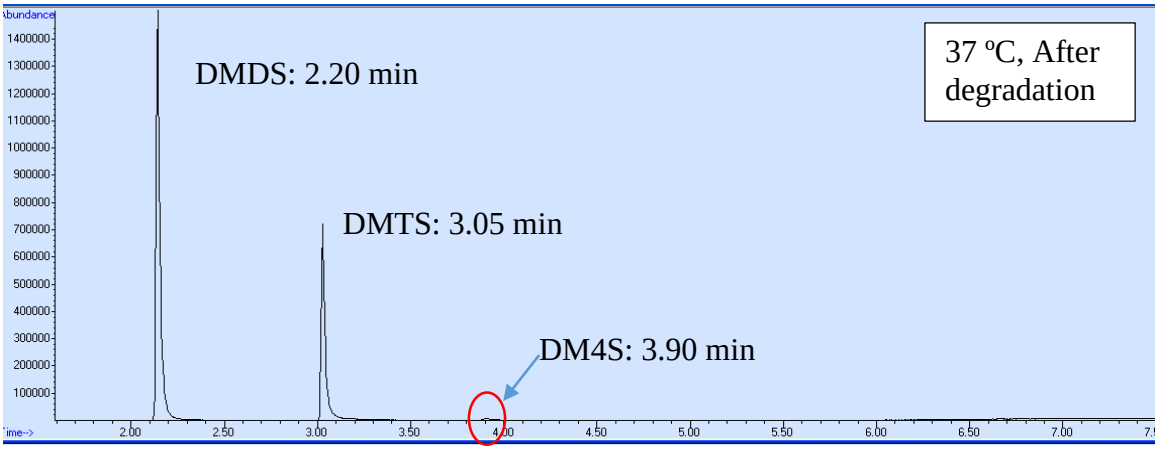


Figure 31. GC Chromatograms of the F3-Formulated DMTS Stored at 37 ºC at Month 11. *600:1 split ratio was used in the SPME method (30 s) and He was used as the carrier gas.*

PART 2

Investigation on the Oxidation Products of DMTS

F3-formulated DMTS was stored in hermetically fire-sealed glass ampules. During storage a small volume of air was contained in the ampule which could lead for oxidation of DMTS. Therefore, to investigate the possible products of DMTS after oxidation, a second project was set up.

Preparation of DMTS and mCPBA was done in CH_2Cl_2, in which they had reasonable solubility. The incubation period was elongated for one week, because two hours after the initiation of the reaction did not produce the expected amount of product as mentioned in the reference.[48]

The prepared solutions of the oxidation reaction were analyzed in HPLC and the following chromatograms were obtained (Figure 32). The pH of the carbonate buffere solution was 9.98 after preparation. This was the suitable pH for removal of biproducts from the reaction mixture such as 3-chlorobenzoic acid (3CBA). On day 0, which is before the reaction began, the DMTS solution prepared with CH_2Cl_2 gave the first chromatogram

shown in the figure (Figure 32). There was a higher amount of DMTS in the reactant giving 0.01 mol/L concentration. DMDS did not use as an internal standard since it appeared as one of the degradation product in long-term storage stability study. Starting from the day 1 the reaction mixture was analyzed in three solution parallels per day. All the chromatograms were obtained from the first parallel solution from each day.

According to the chromatograms obtained from each day during the reaction time of the samples in HPLC-UV/Vis analysis, six significant peaks were able to observe and three of them were changing with the reaction time. The observed peaks were labelled as follows with their retention times. 3CBA (2.2 min), New peak 4 (3.7 min), DMTS peak (10.0 min), New peak 1 (13.7 min) and New peak 2 (20.0 min) in order (Figure 32). The peak numbering kept constant between the two studies.

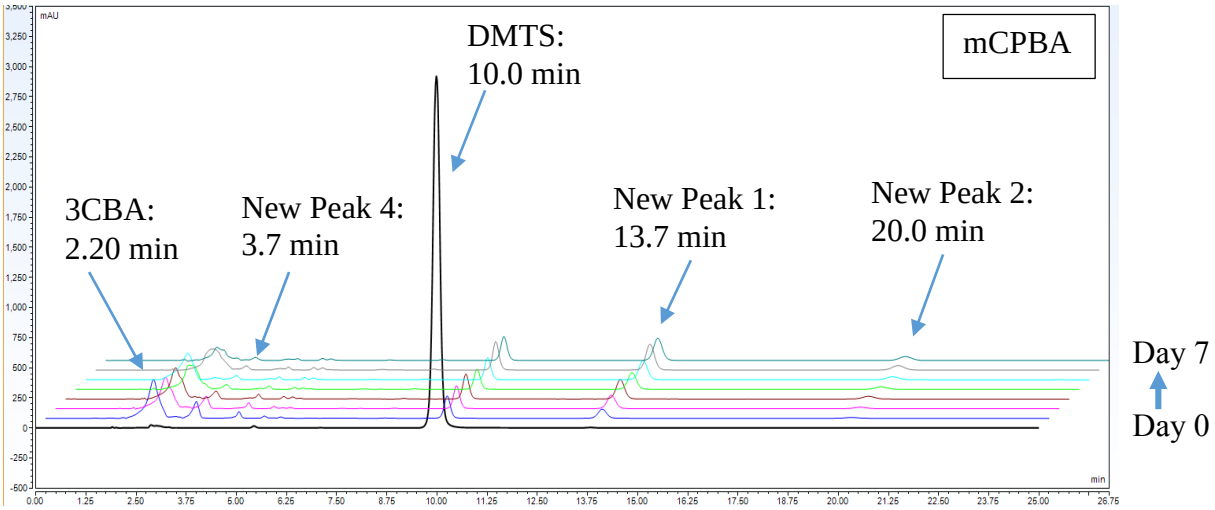


Figure 32. Chromatograms of the HPLC-UV/Vis Analyzed Samples of DMTS Reaction Mixture with mCPBA. *The X axis is retention time and Y axis is mAU*min. Mobile phase was ACN:H_2O = 60:40.*

Therefore, there is a huge difference in peak areas of DMTS in Day 0 sample and rest of the samples.

Identification of the Oxidized Products of the Reaction DMTS with mCPBA

Identification of above observed peaks were carried out with GC-MS and HPLC – UV/Vis. Industrially available samples were used for confirmation. With the help of GC-MS results 4 components were able to identified. The identified components are DMTS at 3.05 min, SMMTS at 3.37 min DM4S at 3.93 min and 3CBA at 4.25 min (Figure 33). The HPLC chromatogram analysis suggested that the peaks observed at 2.2 min is 3-chlorobenzoic acid, peak at 5.0 min is CH_2Cl_2 (Figure 34). The broad peak were not seen earlier since 2.60 min solvent delay was used and were not analyzed.

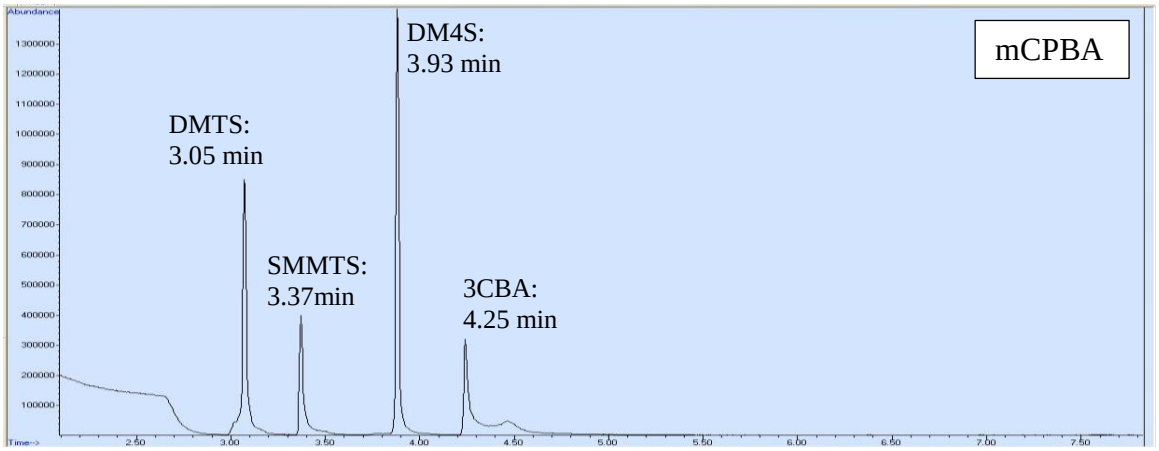


Figure 33. Chromatogram of the GC-MS Analyzed Sample of the DMTS Oxidation Reaction Mixture with mCPBA at Day 7.

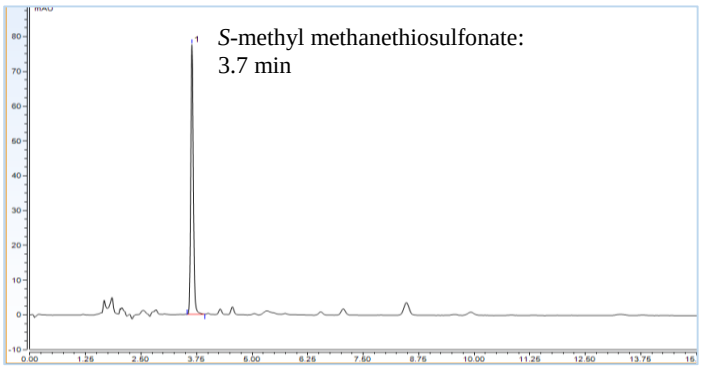


Figure 34. HPLC-UV/Vis Chromatogram of Analyzed Sample of the Commercially Available SMMTS in ACN. *Mobile phase was ACN:H_2O = 60:40.*

The oxidation reaction of DMTS with mCPBA is fast. Since the *S*-methyl methanethiosulfonate is an intermediate product of the reaction its amount was decreased with time. However, the DM4S and product 2 showed increase peak area with time. It seems that the continuous reaction favors the production of more DM4S and product 2 in the long run.

The increase of the observed peaks were plotted against the reaction time and the following graphs were obtained. The statistical analysis of peak areas were conducted over the products which DM4S, DM5S and reactant DMTS (Figure 35). Similarly, the development of peak areas of DM4S and DM5S was clearly shown in the stacked graph of chromatograms. Decrease of peak height and areas of SMMTS may due to precipitation observed during the study period (Figure 32).

DMDS was not used as an internal standard for the quantitative analysis, after we realized that it was also one of the degradation products. After this realization, peak areas were reported either as absolute peak areas or as relative peak ares (ratio of the peak area to the initial DMTS peak area) as shown in Equation 3. It is of significance that we were not able to identify DMDS as one of the oxidation reaction product. We suspected that the oxidation of DMTS may produce DMDS similar to stability study results. But according to the results it might be the DMDS that has oxidized to SMMTS by mCPBA by attaching two oxygen atoms to one of the sulfur atoms.

$$Relative\ Peak\ Area = \frac{Area\ of\ the\ Component\ in\ Spectrum\ with\ Time}{Area\ of\ the\ Initial\ DMTS\ Peak\ in\ Spectrum} \times 100\%$$

Equation 5: Formula for the Calculation of Relative Peak Area.

The initial relative peak area has shown as 100% (m/m), and the Y axis had to be cut down to show the rest of the components.

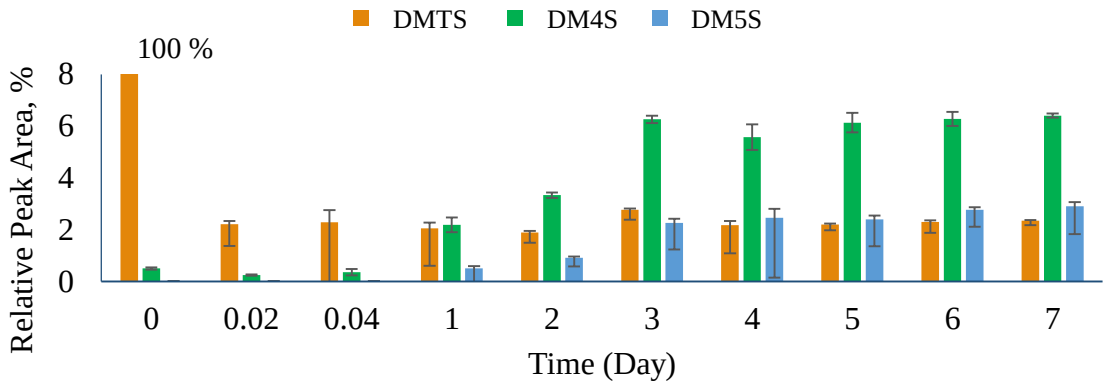


Figure 35. Growing Concentration of Products of DM4S and DM5S; and Reduction in the Concentration of DMTS during its Reaction with mCPBA.

TLC Analysis of Reaction mixture of DMTS with mCPBA

DMTS and other polysulfide molecules are linear and they are non-polar. Therefore, they elute from the reverse phase HPLC column in the order of increasing non-polar characteristic. The oxidized products contain oxygen which makes the molecule polar, therefore they elute from the column early, almost with the solvent phase. The reaction mixture was separated in TLC using their polar characteristics. The non-polar solvent, hexane used as the mobile phase and polar silica TLC plate used as the stationary phase.

The separated reaction mixture on F_{254}-impregnated TLC plate is significantly visible under UV-light (Figure 36). The marked polar spot was extracted using ACN and it was analyzed in GC-MS for identification of components. GC chromatogram confirmed the presence of SMMTS and mCBA in the polar spot (Figure 37) with other several other unidentified compounds. Also there are some components which could not identified by the MS library. Secondly the non-polar spot was extracted using acetone and it was

analyzed in GC-MS. According to the MS library it confirmed the observation of DMTS and DM4S in the non-polar spot as components (Figure 38).

Figure 36. Observation of Polar and Non-Polar Spots on TLC Plate under UV-Light.

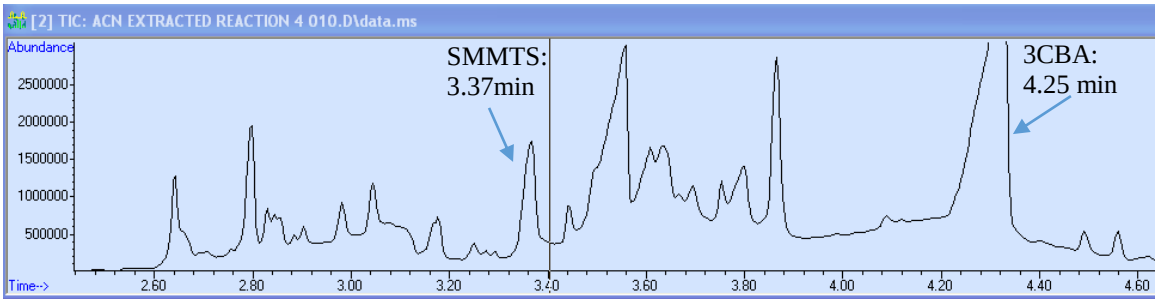


Figure 37. GC-MS Chromatogram of the Analyzed Polar Spot.

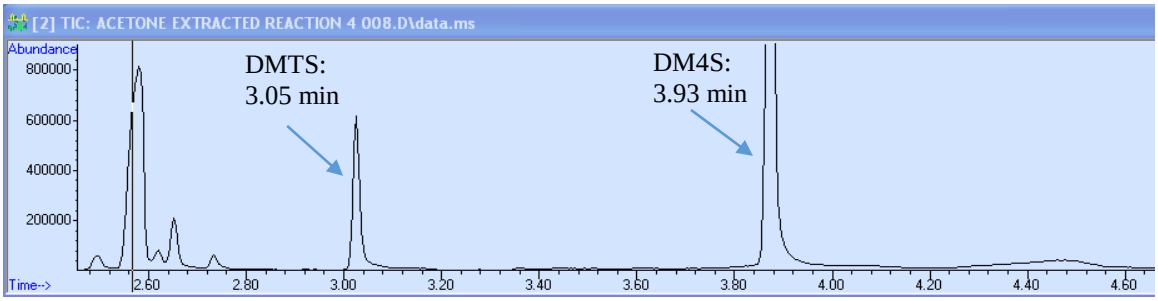


Figure 38. GC-MS Chromatogram of the Analyzed Non-polar Spot.

Identification of the Oxidized Products of the Reaction DMTS with H_2O_2

GC-MS analysis were not carried out for the reaction with H_2O_2, because water used as a solvent. Application of water may damage the GC column due to ability of dissolving many salts. Since the amounts of products from the H_2O_2 used oxidation reaction were less, the TLC plates were not used for extraction for GC-MS analysis. Therefore, quantitative

and qualitative analysis were carried-out for this reaction with H_2O_2 only in HPLC-UV/Vis. According to the HPLC chromatogram analysis, DM4S and DM5S have identified as the significant products (Figure 39), that absorb at the detection wavelength.

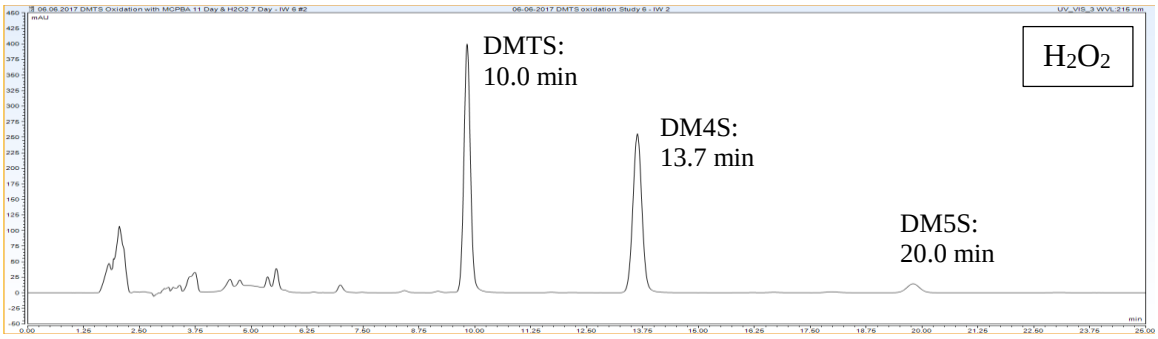


Figure 39. HPLC Spectrum of the Reaction of DMTS and H_2O_2. *Mobile phase was ACN:H_2O = 60:40.*

A similar method was used to quantify the products of the H_2O_2 reaction as the earlier mCPBA reaction (Figure 40). The amounts of products of the H_2O_2 reaction mixture was significantly lower than that of the mCPBA reaction (Figure 35). All the products obtained were less than remaining amount of DMTS after the reaction.

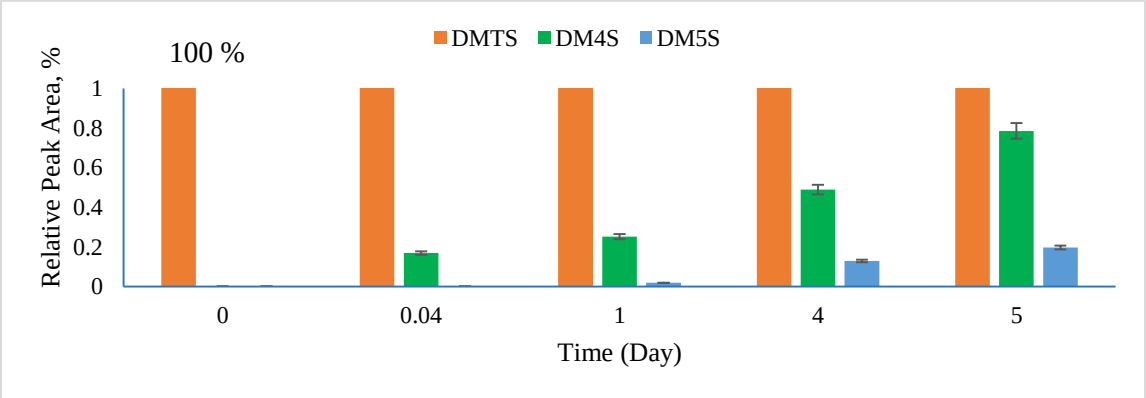


Figure 40. Increase of Products of DM4S and DM5S; and the Decrease of the Reactant DMTS in the Oxidation Reaction with H_2O_2.

HPLC-UV/Vis Spectral Comparison of the Reactions of DMTS with mCPBA or H_2O_2

There is no significant data about development of SMMTS in this reaction mixture. Also the amount of SMMTS has gone down gradually as the mCPBA reaction proceeds.

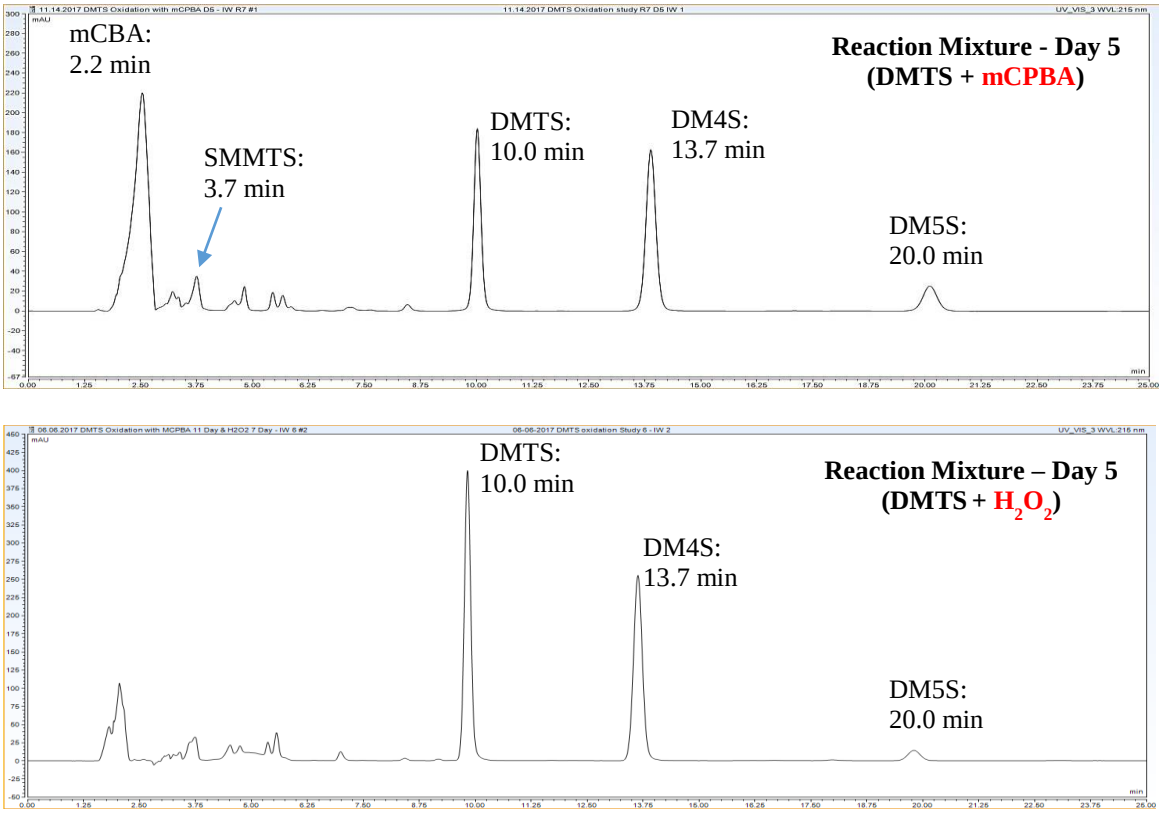


Figure 41. HPLC Spectrum Comparison for Reaction Mixtures of mCPBA and H_2O_2 with DMTS. *Mobile phase was ACN:H_2O = 60:40.*

Comparison between HPLC Methods Used in the Storage Stability Study and the Oxidation Study

Stability Study Sample Analysis with Oxidation Study Method

One-year-old stability study samples were analyzed using oxidation study instrumentation method which uses 60% (v/v) ACN and 40% (v/v) HPLC grade water. Each of the methods have different analysis times which is 18 min and 26 min. Change in

the peak retention times in each of the spectra are due to change in the solvent ratio used in the HPLC methods. According to the stability instrument method the components have lower retention times than oxidation instrumentation method (Figure 42).

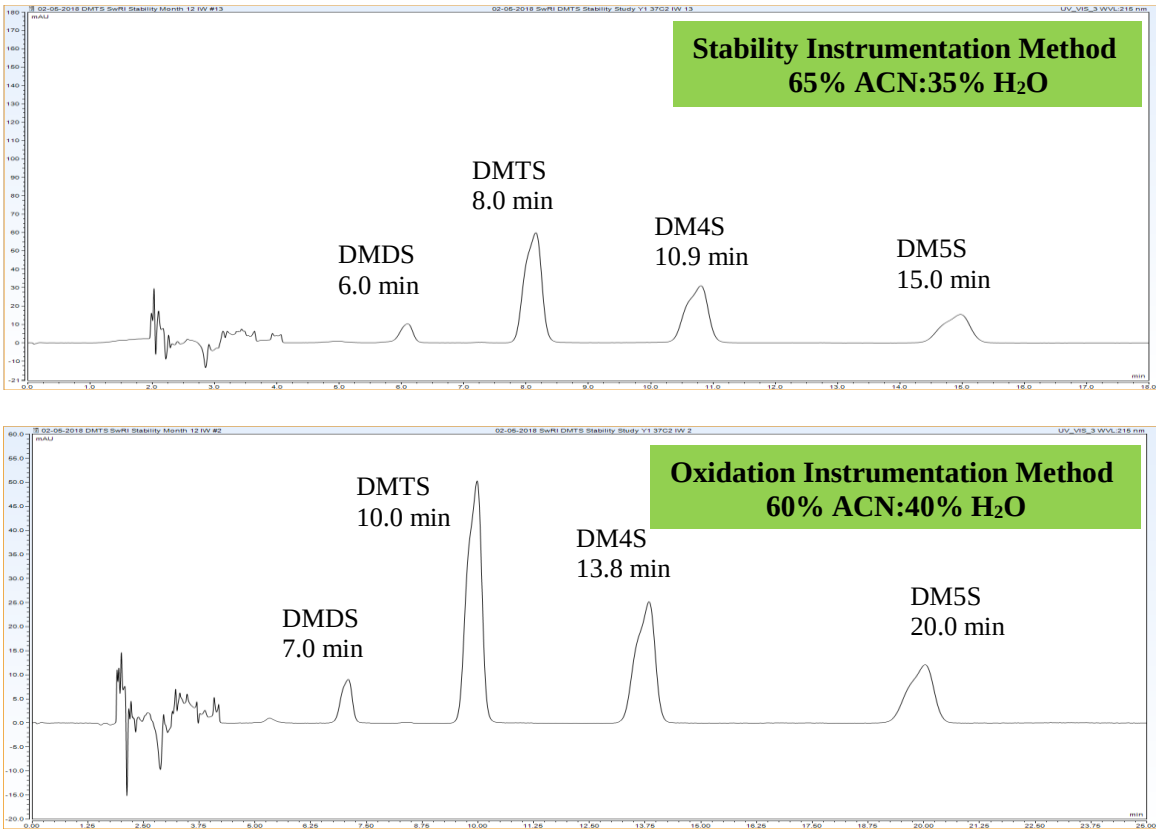


Figure 42. HPLC Spectrums of Stability Study Samples at 37 ºC after One Year. *Samples were analyzed using two different HPLC methods, which used different solvent ratios as mobile phase.*

Analysis and Comparison of the Oxidation Products of DMTS and the F3-Formulated DMTS.

It is necessary to reconcile the retention times for peaks arising from the same compounds in the two different HPLC methods that were employed. Therefore, the samples prepared for oxidation were analyzed using HPLC method used in stability study

to see the change in retention time of each component peak. At the same time, F3-formulated DMTS and pure DMTS were reacted with mCPBA to understand the strength of the formulation during oxidation reaction. Similar concentrations were prepared for the analysis for comparison purposes. After a one-week reaction period, samples were analyzed by HPLC-UV/Vis and similar products were observed. Also, they have shown similar retention times for the components as long-term storage stability study. Same number of moles (0.0005 mol) of pure DMTS, F3-formulated DMTS and mCPBA were used in this experiment to understand the effect of F3-formulation during the reaction with mCPBA. The stoichiometry of the reaction between DMTS and mCPBA did not investigate during this study since it has shown set of reaction during the oxidation of DMTS. In the reaction with pure DMTS, slightly higher amount of DMTS had been reacted and higher amount of DM4S, DM5S have been produced than F3-formulated DMTS reaction. Since the reaction mixture was analyzed after one week the amount of SMMTS was very low and were not used in comparison. In contrast, in the reaction with F3-formulated DMTS, less amount of initial DMTS has reacted with mCPBA compared to pure DMTS reaction, and lesser amounts of DM4S, DM5S and no SMMTS were identified. There might SMMTS present in the F3-formulated DMTS reaction and acted similar to pure DMTS reaction. This suggests that F3-formulation provides certain level of protection over DMTS, controlling the reaction availabilities of DMTS with mCPBA (Figure 43). The peak areas were analyzed comparative to the initial amount of DMTS before each reactions and no replicates were used, therefore no standard deviations were calculated (Figure 44).

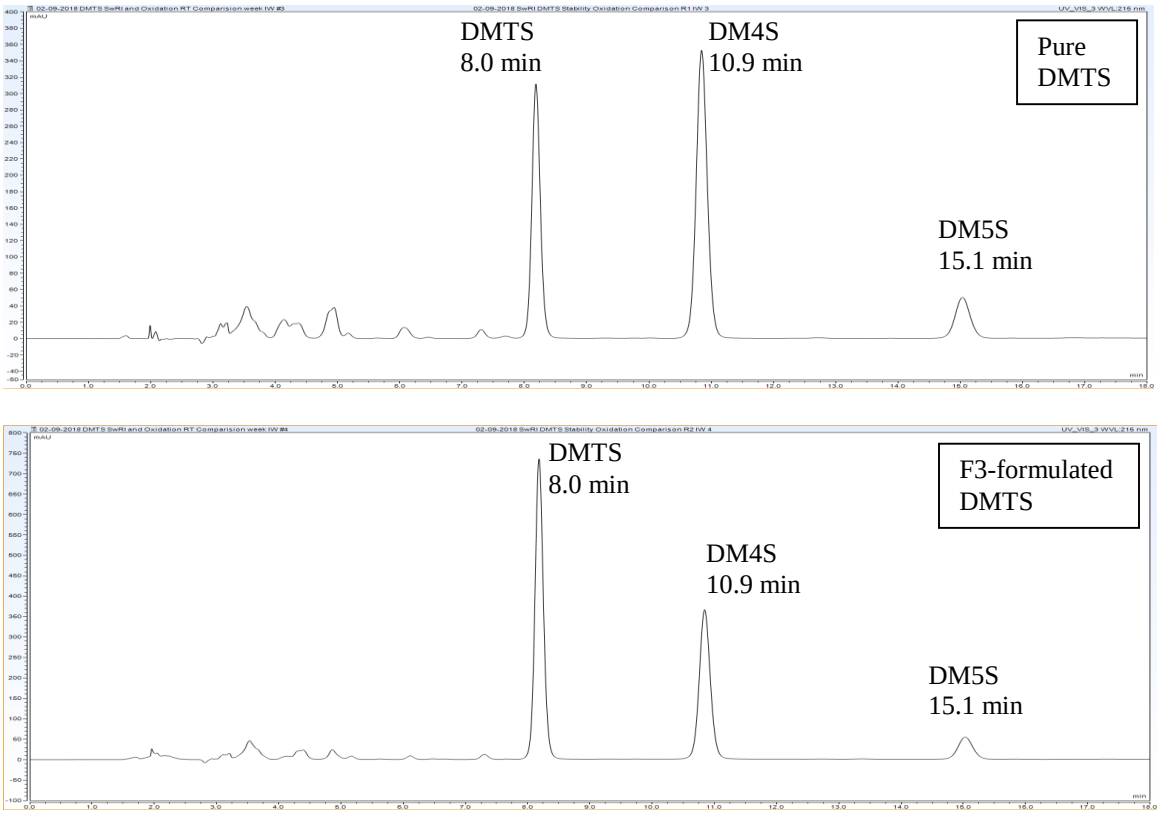


Figure 43. HPLC-UV/Vis Spectra of the Two Reaction Mixtures with mCPBA. *Mobile phase was ACN:H_2O = 65:35.*

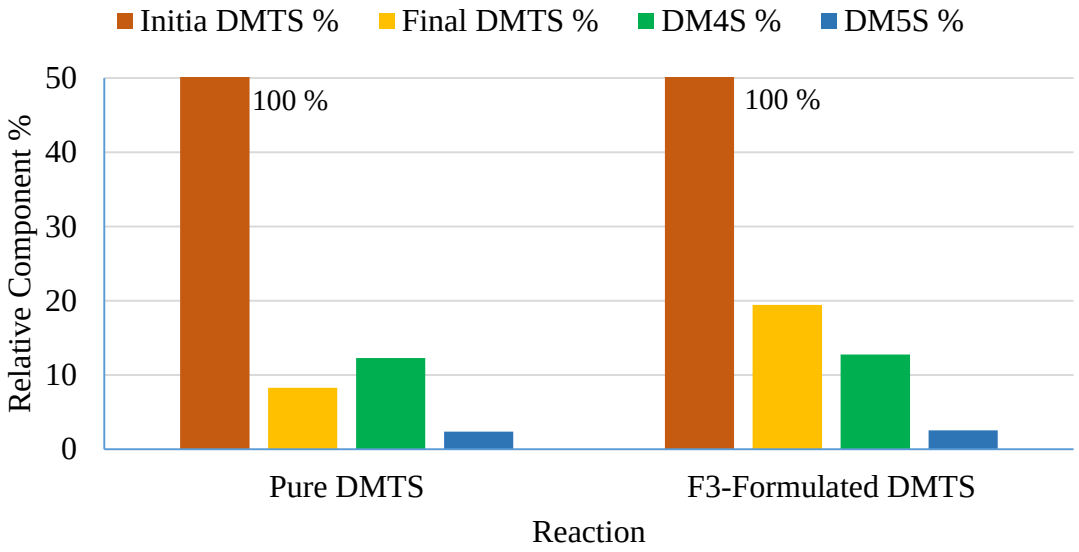


Figure 44. Product Amount Comparison of mCPBA Reactions with Pure DMTS and F3-Formulated DMTS.

CHAPTER IV

CONCLUSION

PART 1

Long-Term Storage Stability of the F3-Formulated DMTS

F3-formulated DMTS showed high long-term storage stability within a one-year period. The samples stored at 4 and 22 ºC displayed 100% (M/M) stability over a 12-month study. However, the samples stored at 37 ºC showed 100% (M/M) stability only over a 5-month period and gradually decreased to 30% (M/M) of initial concentration at the end of the study. The F3-formulation itself provided better protection for DMTS by lowering reaction rate even in the presence of strong oxidizing agents. Therefore, it can be concluded that the hermetically fire-sealed glass ampules can provide significantly better protection for F3-formulated DMTS in the long run.

Investigation on the Degradation Products of the F3-Formulated DMTS

Due to the degradation of F3-formulated DMTS there were several degradation products observed. The three major identified degradation products are DMDS, DM4S and DM5S which have relative retention times of 6.0 min, 10.9 min and 15.1 min respectively. Dimethyl polysulfides were recognized as the products of the disproportionation reaction of DMTS at high temperatures. We can conclude that in our experiments DMDS, DM4S and DM5S formed during the disproportionation of DMTS.

PART 2

Identification of the Oxidized Products of DMTS in the Presence of Strong Oxidizing Agents

Reaction with mCPBA.

The oxidation reaction with mCPBA yielded DM4S, DM5S and SMMTS, but no DMDS as a product.

Reaction with H_2O_2.

The oxidation reaction with H_2O_2 showed the formation of DM4S and DM5S, but there was no observation of SMMTS. This may be due to the lower oxidation strength of the 3% H_2O_2.

Comparison of the Products of the Long-term Storage Stability Study and the Oxidation Study

The products identified from the long-term storage stability study and the oxidation reaction showed an overlap: both DM4S and DM5S were found in both. Additionally, DMDS was observed as a product during the degradation of DMTS but did not appear during the oxidation study. The results observed from stability study matched with the literature for disproportionation. The production of SMMTS and the absence of DMDS in oxidation with mCPBA can be mentioned as the difference between the two studies. The results of the TLC analysis suggested the feasibility of preparative chromatography for the identification of trace amounts of compounds.

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APPENDIX A

Glossary

CN: cyanide

CN^-: cyanide anion

TS: thiosulfate

Rh: Rhodanese

SD: sulfur donor

DMS: dimethyl sulfide

DMTS: dimethyl trisulfide

DMDS: dimethyl disulfide

DM4S: dimethyl tetrasulfide

DM5S: dimethyl pentasulfide

SMMTS: *S*-methyl methanethiosulfonate

mCPBA: meta-chloroperbenzoic acid

mCBA/3CBA: meta-chlorobenzoic acid

poly 80: polyoxyethylenesorbitan monooleate

span 80: sorbitan monoenolate

HPLC: high pressure liquid chromatography

GC-MS: gas chromatography mass spectrometry

TLC: thin layer chromatography

IM: intramuscular

IV: intravenous

S.D.: sample standard deviation

(*m/m*): mass/mass ratio

(M/M): concentration/concentration ratio

APPENDIX B

Data and the calculations used to obtain the Figure 23.

	Day 0						
	DMDS	DMTS	Ratio	Avg. Rat.	%	Avg. %	% RSD
	12.594	41.968	3.33238		101.6822		
	12.334	39.34	3.189557		97.32417		
4	12.756	42.22	3.309815	3.277251	100.9936	100	2.34277
	12.594	41.968	3.33238		101.6822		
	12.334	39.34	3.189557		97.32417		
22	12.756	42.22	3.309815	3.277251	100.9936	100	2.34277
	12.594	41.968	3.33238		101.6822		
	12.334	39.34	3.189557		97.32417		
37	12.756	42.22	3.309815	3.277251	100.9936	100	2.34277

			Day 6				
DMDS	DMTS	Ratio	Avg. Rat.	%	Avg. %	% RSD	
14.314	45.716	3.193796		97.45352			
14.522	46.985	3.235436		98.72408			
14.417	47.3	3.280849	3.236694	100.1098	98.76246	1.328552	
14.431	46.151	3.198046		97.58319			
14.414	46.396	3.218815		98.21692			
14.498	46.737	3.223686	3.213516	98.36555	98.05522	0.415494	
14.277	46.27	3.240877		98.89011			
14.379	46.098	3.205925		97.82361			
14.358	46.767	3.257209	3.23467	99.38844	98.70072	0.799418	

			Week 1				
DMDS	DMTS	Ratio	Avg. Rat.	%	Avg. %	% RSD	
14.224	46.01	3.234674		98.70083			
14.455	45.227	3.128814		95.47067			
14.018	47.96	3.421315	3.261601	104.3959	99.52247	4.518983	
14.449	45.474	3.147207		96.03193			
13.691	45.721	3.339493		101.8992			
14.491	47.224	3.25885	3.248517	99.43854	99.12323	2.946324	
14.538	46.041	3.166942		96.6341			
14.313	46.711	3.263537		99.58153			
14.468	50.726	3.506082	3.312187	106.9824	101.066	5.331485	

Week 2						
DMDS	DMTS	Ratio	Avg. Rat.	%	Avg. %	% RSD
13.972	47.267	3.38298		103.2262		
14.084	48.404	3.436808		104.8686		
14.159	47.036	3.321986	3.380591	101.365	103.1533	1.752936
13.953	47.452	3.400846		103.7713		
14.041	47.625	3.391852		103.4969		
13.991	48.339	3.455007	3.415902	105.4239	104.2307	1.042437
14.308	46.325	3.237699		98.79314		
14.171	46.314	3.268224		99.72455		
14.143	46.451	3.284381	3.263435	100.2176	99.57842	0.723366

Week 3						
DMDS	DMTS	Ratio	Avg. Rat.	%	Avg. %	% RSD
13.699	43.793	3.196803		97.54525		
13.253	45.229	3.412737		104.1341		
13.911	44.788	3.21961	3.276383	98.24119	99.97352	3.619953
14.003	43.02	3.072199		93.74317		
13.972	44.867	3.211208		97.98481		
13.83	47.509	3.435213	3.23954	104.82	98.84931	5.588774
13.93	45.033	3.232807		98.64386		
13.924	45.931	3.298693		100.6543		
13.82	44.815	3.242764	3.258088	98.94769	99.41527	1.083701

Month 1						
DMDS	DMTS	Ratio	Avg. Rat.	%	Avg. %	% RSD
14.129	44.607	3.157124		96.33451		
14.152	45.061	3.184073		97.15682		
14.336	46.088	3.214844	3.185347	98.09575	97.19569	0.881261
14.226	46.673	3.280824		100.109		
14.056	43.889	3.122439		95.27616		
14.078	44.572	3.166075	3.189779	96.60764	97.33094	2.4963
14.297	45.974	3.21564		98.12003		
13.869	42.512	3.065253		93.53124		
14.074	46.886	3.331391	3.204095	101.652	97.76776	4.071826

Month 2						
DMDS	DMTS	Ratio	Avg. Rat.	%	Avg. %	% RSD
13.38	47.671	3.562855		108.7147		
13.385	41.697	3.115204		95.05539		
13.625	45.855	3.365505	3.347854	102.6929	102.1544	6.845586
13.758	47.92	3.483064		106.2801		
13.747	44.346	3.225867		98.43212		
13.897	46.055	3.314025	3.340985	101.1221	101.9448	3.988127
13.714	44.786	3.265714		99.64797		
13.872	44.745	3.225562		98.4228		
13.896	46.744	3.363846	3.285041	102.6423	100.2377	2.170683

Month 3						
DMDS	DMTS	Ratio	Avg. Rat.	%	Avg. %	% RSD
13.254	48.133	3.631583		110.8119		
14.13	45.955	3.2523		99.23866		
14.169	45.385	3.203119	3.362334	97.738	102.5962	7.154451
13.187	43.357	3.287859		100.3237		
13.036	43.409	3.329932		101.6075		
13.454	41.828	3.108964	3.242252	94.86499	98.93206	3.580197
13.598	44.813	3.295558		100.5586		
13.375	47.245	3.532336		107.7835		
13.516	44.788	3.313702	3.380532	101.1123	103.1515	4.021019

Month 4						
DMDS	DMTS	Ratio	Avg. Rat.	%	Avg. %	% RSD
13.254	51.017	3.849178		117.4514		
13.13	42.037	3.201599		97.69162		
13.169	43.093	3.272306	3.441028	99.84912	104.9974	10.83932
13.187	42.041	3.188064		97.27861		
13.036	42.758	3.279994		100.0837		
13.454	51.82	3.851643	3.4399	117.5266	104.963	10.97047
13.298	47.647	3.58302		109.33		
13.375	41.961	3.137271		95.72874		
13.516	42.039	3.110314	3.276868	94.90618	99.98832	8.100618

Month 5						
DMDS	DMTS	Ratio	Avg. Rat.	%	Avg. %	% RSD
13.003	42.904	3.299546		100.6803		
13.087	42.987	3.28471		100.2276		
13.25	46.129	3.481434	3.35523	106.2303	102.3794	3.342656
12.026	43.115	3.585149		109.395		
12.919	40.44	3.130273		95.51521		
13.008	42.696	3.282288	3.33257	100.1537	101.688	7.065951
12.181	47.28	3.881455		118.4363		
12.289	39.963	3.251933		99.22745		
12.146	40.602	3.342829	3.492072	102.001	106.5549	10.38261

Month 6						
DMDS	DMTS	Ratio	Avg. Rat.	%	Avg. %	% RSD
13.357	52.763	3.950213		120.5344		
13.258	45.35	3.420576		104.3733		
13.316	40.485	3.040327	3.470372	92.77066	105.8928	8.204331
13.411	45.548	3.396316		103.6331		
13.367	44.805	3.351911		102.2781		
13.293	41.798	3.144362	3.29753	95.9451	100.6188	4.10383
13.793	41.719	3.02465		92.2923		
13.831	41.865	3.026896		92.36083		
13.799	42.331	3.067686	3.039744	93.60547	92.75286	0.739171

Month 7						
DMDS	DMTS	Ratio	Avg. Rat.	%	Avg. %	% RSD
13.268	51.12	3.852879		117.5644		
13.239	44.268	3.343757		102.0293		
13.233	43.657	3.299101	3.498579	100.6667	106.7535	9.387266
13.05	50.382	3.86069		117.8027		
13.149	49.066	3.731539		113.8618		
13.203	40.672	3.080512	3.55758	93.99683	103.9293	14.04669
15.777	19.582	1.241174		37.87241		
14.151	39.419	2.785598		84.99801		
14.022	38.27	2.729283	2.252018	83.27963	68.71668	26.72574

Month 8						
DMDS	DMTS	Ratio	Avg. Rat.	%	Avg. %	% RSD
62.472	50.638	0.810571		24.73326		
13.93	44.991	3.229792		98.55186		
13.934	44.867	3.219966	2.42011	98.25203	98.40195	0.212014
13.918	49.387	3.548426		108.2745		
13.861	44.533	3.212827		98.03422		
13.892	44.375	3.194284	3.318513	97.46841	101.259	6.082136
14.602	20.47	1.401863		42.77557		
14.551	20.379	1.400522		42.73467		
14.56	20.313	1.395124	1.39917	42.56994	42.69339	0.108854

Month 9						
DMDS	DMTS	Ratio	Avg. Rat.	%	Avg. %	% RSD
12.996	42.957	3.305402		100.859		
12.042	40.301	3.346703		102.1192		
12.98	42.265	3.256163	3.302756	99.35655	100.7782	1.383107
12.975	42.364	3.265048		99.62765		
12.146	40.987	3.374527		102.9682		
12.932	42.357	3.275363	3.304979	99.94241	100.8461	1.844536
12.611	39.887	3.162874		96.50996		
14.13	19.648	1.390517		42.42936		
14.515	19.702	1.357354	1.970248	41.41747	41.92342	0.715514

Month 11						
DMDS	DMTS	Ratio	Avg. Rat.	%	Avg. %	% RSD
13.609	43.324	3.183482		97.13878		
13.713	45.36	3.30781		100.9325		
13.793	47.307	3.429783	3.307025	104.6543	100.9085	3.757806
13.993	49.215	3.517116		107.3191		
13.858	45.535	3.285828		100.2617		
13.761	42.551	3.092144	3.298363	94.35178	100.6442	6.49211
13.745	18.734	1.362968		41.58877		
13.912	17.958	1.290828		39.38753		
13.922	17.866	1.283293	1.312363	39.15759	40.04463	1.342197

Month 12						
DMDS	DMTS	Ratio	Avg. Rat.	%	Avg. %	% RSD
13.609	44.549	3.273495		99.88541		
13.713	45.296	3.303143		100.7901		
13.793	46.942	3.403321	3.326653	103.8468	101.5074	2.075846
13.993	45.355	3.241263		98.9019		
13.858	43.929	3.169938		96.72552		
13.761	43.315	3.147664	3.186288	96.04586	97.22442	1.491955
13.945	17.725	1.271065		38.78449		
13.912	16.836	1.210178		36.92663		
13.922	16.954	1.217785	1.233009	37.15873	37.62328	1.012306

VITA

Indika Kasun Warnakula

Education

2016-present M.S. in chemistry, Sam Houston State University, Huntsville, TX, US

2014-2016 M.S. in Analytical Chemistry, University of Peradeniya, Peradeniya, Sri Lanka

2010–2013 B.Sc. Physical Science, University of Peradeniya, Peradeniya, Sri Lanka

Academic Employment

2016-present Teaching Assistant, Chemistry Department, Sam Houston State University

- Instructed students in the following course labs: General Chemistry I, Introductory Organic and Biochemistry, Organic Chemistry I, Organic Chemistry II, Quantitative Analysis
- Specific work included lab set up and maintenance, evaluation of examinations, assignments, and lab reports. 20 hours per week, each semester.

2015-2016 Postgraduate Teaching assistant at Postgraduate Institute of Science, University of Peradeniya

- Specific duties such as perform laboratory sessions helping postgraduate students with their experiments, evaluating quizzes, examination papers and lab reports, perform tutorial discussions.

Publications

- **Warnakula, I.K**., Ebrahimpour, A., Kiss, M., Thompson, D.E., Rios, C.T., Gaspe Ralalage, R.D., Rockwood, G.A., Petrikovics, I. Storage Stability Studies with the

SwRI Formulated Cyanide Antidote Dimethyl Trisulfide, Society of Toxicology National Meeting, March 11-15, **2018**, San Antonio, Texas. (Abstract # 2304/P649)

- Kiss, M., Sipos, P., **Warnakula, I.K.**, Vergara, M., Rios, C.T., Whiteman, A., Gaspe R., R.D., Rockwood, G.A., Petrikovics, I., Developing and Testing a New Intramuscular Formulation for the Cyanide Antidote Dimethyl Trisulfide. 57th SOT Annual Meeting, March 11-15, **2018**, San Antonio, TX. (Abstract # 2291/P235)
- **Warnakula, I.K.**, Kiss, M., Rios, C.T., Vergara, M., Gaspe Ralalage, R.D., Whiteman, A.C., Petrikovics, I. Storage Stability Studies with the SwRI Formulated Cyanide Antidote Dimethyl Trisulfide, Texas Academy of Science Meeting, March 2-4, **2018**, Midland, Texas. (Abstract 020.158G)
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- Riso, C.T.S., Vergara, M.N., Ebrahimpour, A., Kiss, M., **Warnakula, I.K.**, Gaspe Ralalage, R.D., Hewa R, C.C., Barrera, I., Petrikovics, I., In vitro and in vivo characterization of the cyanide antidote SDX6F2, *ACS South West Regional Meeting*, October 29- November 01, **2017**, Lubbock, Texas (Abstract # 2821909)
- Gaspe Ralalage, R.D., Hewa R, C.C., **Warnakula I.K.**, Rios, C.T.S., Kiss, M., Roy, R.J., Baca, W., Ebrahimpour, A., Petrikovics, I., Comparision of three different cyanide antidote candidate sulfur donor molecules in vitro and in vivo,

ACS South West Regional Meeting, October 29- November 01, **2017**, Lubbock, Texas (Abstract # 2821922)

- Hewa R., C.C.; Gaspe R., R.D.; **Warnakula, I.K.**; Preethika, D.K.N.; Baca, W.; Kiss, L.; Ebrahimpour, A.; Petrikovics, I.; Characterization of the cyanide antidote candidate SAX6 in two formulation forms: Blood Brain Barrier (BBB) penetration, Size distribution, *In vivo* efficacy and Pharmacokinetics. *56th SOT Annual Meeting*, March 12-16, **2017**, Baltimore, MD. (Abstract # 2052/P214)

- Hewa R, C. C.; Gaspe Ralalage, R. D.; **Warnakula, I.K.**; Ebrahimpour, Afshin; Petrikovics, I. "Modelling Blood Brain Barrier (BBB) penetration by in-vitro parallel artificial membrane permeability study with the newly formulated cyanide (CN) antidote, dimethyl trisulfide (DMTS), Texas Academy of Science Annual Meeting, March, 3-5, **2017,** Belton, Texas. (Abstract#: 017.044G)

- Carpenter, M.; Kefer, E.; **Warnakula, I.K.**; Barrera, I.; Gaspe Ralalage, R.D.; Ebrahimpour, A.; Petrikovics, I. Blood Partitioning and Elimination Study with SMDEX, a new Cyanide Antidote, *Texas Academy of Science Annual Meeting*, March, 3-5, **2017,** Belton, Texas. (Abstract#: 036.203G)

- Priyantha, N., **Warnakula, I.K.**, Mallikarathna, W.A.S.A., Removal of Cu(II) by Humic Acid Extracted from Muthurajawela Peat, *Fifth International Symposium on Water Quality and Human Health*, August **2016,** Postgraduate Institute of Science, University of Peradeniya, Peradeniya, Sri Lanka.

- Wickramasooriya, S., **Warnakula, I.K.**, Priyantha, N., Enhancement of Cu(II) Removal by Humic Acid Extracted form Muthurajawela Peat, Research Publication on progress - **2016**
- **Warnakula I.K.**, Removal of Cu(II) and Cr(III) ions by humic acid extracted from Muthurajawela Peat, M.Sc. thesis - **2016**
- Priyantha, N., **Warnakula, I.K.**, Wickramasooriya, M.U.S., Improvement of Water Quality through Metal Ion Adsorption by Humic acid Extracted from Muthurajawala Peat: A Case Study of Cu(II) Adsorption, *Fourth International Symposium on Water Quality and Human Health*, August **2015,** Postgraduate Institute of Science, University of Peradeniya, Peradeniya, Sri Lanka.
- Web base Exam practicing system for local students using PHP, HTML 5, Angular.JS, MySQL, Wamp Server and Jquery - **2013**.

Awards and Honors

- SUSU Graduate Student Excellence in Research 2018 Award – Summer 2018
- SHSU College of Science Department of Chemistry Robert A. Welch Scholarship for Research – Summer 2018
- SHSU College of Science and Engineering Technology Travel Award – Spring 2018
- SHSU College of Science and Engineering Technology (COSET) Special Scholarship – Spring 2018
- SHSU College of Science and Engineering Technology (COSET) Special Scholarship – Fall 2017
- SHSU College of Science Travel Award – Fall 2017
- SHSU College of Science Department of Chemistry Robert A. Welch Scholarship for Research – Summer 2017